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REGULATION OF CHOLINERGIC NEUROTRANSMISSION IN THE PORCINE AND HUMAN GASTROINTESTINAL TRACT

THESIS SUBMITTED AS PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR IN MEDICAL SCIENCES

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LIST OF ABBREVIATIONS

4-DAMP	4-diphenylacetoxy-*N*-methylpiperidine methiodide
5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
Bq	becquerel
Ci	Curie
CM	circular muscle
CNS	central nervous system
Cyclic AMP	adenosine 3'5' cyclic monophosphate
Cyclic GMP	guanosine 3'5' cyclic monophosphate
DAG	diacylglycerol
DMSO	dimethylsulfoxide
EFS	electrical field stimulation
eNOS	endothelial nitric oxide synthase
ENS	enteric nervous system
GI	gastrointestinal
GMC	giant migrating contraction
HPLC	high performance liquid chromatography
Hz	hertz
IC_{50}	concentration of an antagonist responsible for 50% of its maximal effect
ICC	interstitial cells of Cajal
iNOS	inducible nitric oxide synthase
IP_3	inositol-1,4,5-trisphosphate
IPAN	intrinsic primary afferent neuron
KCl	potassium chloride
LMMP	longitudinal muscle-myenteric plexus
L-NAME	L-N^G-nitroarginine methyl ester
L-NNA	L-N^G-nitroarginine
MMC	migrating motor complexes
MT-3	mamba toxin-3
NANC	non-adrenergic non-cholinergic
NO	nitric oxide

NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
ODQ	1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one
P	probability
PACAP	pituitary adenylate cyclase-activating polypeptide
p-F-HHSiD	*para*-fluoro-hexahydro-sila-difenidol hydrochloride
PK	protein kinase
PSS	physiological salt solution
r	Pearson correlation coefficient
s.e.mean	standard error of the mean
SIN-1	3-morpholino-sydnonimine
SNP	sodium nitroprusside
TR	total radioactivity
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide

CONTENT

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INTRODUCTION AND AIMS

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Gastrointestinal (GI) motility is under neuronal and hormonal control. The cholinergic neurones are the most important between the neurones, that release a contractile neurotransmitter. Different types of neurones release other contractile or relaxant neurotransmitters. It is known from studies in the peripheral and central nervous system, that the release of acetylcholine from cholinergic nerve endings can be modified *via* presynaptic auto- and heteroreceptors, whereby the release of acetylcholine can be increased or inhibited. In the GI tract, the presynaptic regulation of acetylcholine release has not been studied in a systematic way. Especially the possible presynaptic regulation of GI acetylcholine release by the most important relaxant neurotransmitter nitric oxide (NO) has not been clarified. Some studies were performed to investigate this interaction between acetylcholine and NO, but most were functional studies measuring smooth muscle contraction as outcome. These studies do not allow to localize the interaction between acetylcholine and another substance with certainty at the pre- or postsynaptic level. To investigate the presynaptic regulation of acetylcholine release, a technique measuring acetylcholine release directly must be applied.

The aim of this thesis was to study the presynaptic regulation of acetylcholine release in the porcine and human GI tract. The first tissue examined was the gastric fundus, where cholinergic neurones play a role in the development of the pressure gradient, that pushes ingested material to the distal stomach. This part of the stomach also contains an important nitrergic innervation, allowing to concentrate on the possible presynaptic interaction between acetylcholine and NO. The muscarinic autoreceptors, inhibiting acetylcholine release, as well as the postsynaptic muscarinic receptors mediating cholinergic contractions were pharmacologically characterized. In view of the interest for gastrokinetic agents, the influence of the 5-HT_4-receptor agonist prucalopride was investigated. This agent also stimulates colonic motility leading us to evaluate its influence on acetylcholine release in the colon.

Chapter 1 gives a literature survey about the innervation of the GI tract, and the neuronal contribution in motility regulation of stomach and colon, the two tissues under study. This chapter also summarizes previous studies with regard to presynaptic modulation of acetylcholine release in different parts of the GI tract.

Chapter 2 gives the results of functional experiments, performed to study the cholinergic-nitrergic interaction in circular muscle strips of pig gastric fundus. The pig was

selected as experimental animal as it has been proposed that this animal is a good non-primate model for studying human digestive function in view of the similarity of the morphology and physiology of the GI tracts (Miller & Ullrey, 1987). The pig gastric fundus is innervated by both excitatory cholinergic and inhibitory non-adrenergic non-cholinergic (NANC) neurones (Ohga & Taneike, 1977; Miyazaki *et al.*, 1991), and NO is the most important NANC neurotransmitter (Lefebvre *et al.*, 1995). Although the experiments suggested that NO interacted with acetylcholine by functional antagonism at the postsynaptic level, a presynaptic effect of NO could not be excluded. A method was therefore introduced to measure acetylcholine release from the cholinergic nerve endings after incubation of the tissue with [^{3}H]-choline. **Chapter 3** describes in detail how the experimental conditions were adapted to measure [^{3}H]-acetylcholine release in the pig gastric fundus. This study allowed to determine more precisely whether NO interferes with the release of [^{3}H]-acetylcholine from cholinergic neurones innervating the circular muscle layer of pig gastric fundus. As it has been demonstrated that presynaptic α_2-adrenoceptors (De Ponti *et al.*, 1996) and muscarinic auto-receptors (Starke *et al.*, 1989; Grimm *et al.*, 1994) can interfere with acetylcholine release, a series of experiments was performed to study whether these receptors are present on the cholinergic nerve endings innervating the circular muscle layer of the pig gastric fundus.

The previous experiments demonstrated the presence of inhibitory muscarinic receptors on the cholinergic nerve endings in pig gastric fundus. In **Chapter 4**, an attempt was made to pharmacologically characterize these presynaptic muscarinic autoreceptors by using a series of subtype-preferring muscarinic antagonists. A second goal was the characterization of the postsynaptic muscarinic receptors, responsible for the contraction of the tissue by the released acetylcholine after activation of the cholinergic neurones. Both functional and release experiments were used to pharmacologically characterize the muscarinic receptors.

As cholinergic neurones innervate human gastric fundus (Parkman *et al.*, 1999; Tonini *et al.*, 2000), it should be possible to measure [^{3}H]-acetylcholine release from cholinergic neurones innervating the circular muscle layer of human gastric fundus. The same protocol as used for the pig gastric fundus was used to study the release of [^{3}H]-acetylcholine in human proximal stomach; the results are given in **Chapter 5**. In human gastric fundus, both NO and VIP contribute to NANC relaxations (Tonini *et al.*, 2000), and the influence of NO and VIP on [^{3}H]-acetylcholine from cholinergic neurones innervating the circular muscle layer was studied. The presence of α_2-adrenoceptors and muscarinic autoreceptors on the cholinergic neurones was also investigated. As it is demonstrated that stimulation of 5-HT$_4$-receptors

increases human stomach emptying (Johnson, 1989), the presence of facilitatory 5-HT_4-receptors on the cholinergic nerve endings was studied.

Because of our interest in 5-HT_4-receptors, the human colon was studied in **Chapter 6** as it is known that stimulation of 5-HT_4-receptors increases human colonic motility *in vivo* (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999, 2001), but the precise mechanism of this effect is not yet known. In human colon, 5-HT_4-receptors have been demonstrated on circular muscle cells, causing relaxation (Prins *et al.*, 2000b) while 5-HT_4-receptors are also present on the cholinergic nerve endings innervating the longitudinal muscle layer, facilitating cholinergic contractions (Prins *et al.*, 2000a). However, the latter can very probably not explain why 5-HT_4-receptor agonists accelerate human colonic transit as the contraction of the longitudinal muscle layer can not provide sufficient force to promote colonic transit. Facilitatory 5-HT_4-receptors on cholinergic nerve endings innervating the circular muscle layer might explain the increase in transit. For this reason, the presence of stimulatory presynaptic 5-HT_4-receptors on cholinergic nerve endings innervating the circular muscle layer in human colon was investigated, by means of contractility and release experiments.

Chapter 7 discusses the results, leading to the conclusion.

References

BOURAS, E.P., CAMILLERI, M., BURTON, D.D. & MCKINZIE, S. (1999). Selective stimulation of colonic transit by the benzofuran $5HT_4$ agonist, prucalopride, in healthy humans. *Gut*, **44**, 682-686

BOURAS, E.P., CAMILLERI, M., BURTON, D.D., THOMFORDE, G., MCKINZIE, S. & ZINSMEISTER, A.R. (2001). Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. *Gastroenterology*, **120**, 354-360

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.*, **69**, 59-78

EMMANUEL, A.V., KAMM, M.A., ROY, A.J. & ANTONELLI, K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut*, **42**, 511-516

GRIMM, U., MOSER, E., MUTSCHLER, M.E. & LAMBRECHT, G. (1994). Muscarinic receptors: focus on presynaptic mechanisms and recently developed novel agonists and antagonists. *Pharmazie*, **49**, 711-726

JOHNSON, A.G. (1989). The effects of cisapride on antroduodenal co-ordination and gastric emptying. *Scand. J. Gastroenterol.*, **24**, 36-43

LEFEBVRE, R.A., SMITS, G.J.M. & TIMMERMANS, J.-P. (1995). Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. *Br. J. Pharmacol.*, **116**, 2017-2026

MILLER, E.R. & ULLREY, D.E. (1987). The pig as a model for human nutrition. *Ann. Rev. Nutr.*, **7**, 361-382

MIYAZAKI, H., KOYAMA, I., NAKAMURA, H., TANEIKE, T. & OHGA, A. (1991). Regional differences in cholinergic innervation and drug sensitivity in the smooth muscle of pig stomach. *J. Auton. Pharmacol.*, **11**, 255-265

OHGA, A. & TANEIKE, T. (1977). Dissimilarity between the responses to adenosine triphosphate or its related compounds and non-adrenergic inhibitory nerve stimulation in the longitudinal smooth muscle of pig stomach. *Br. J. Pharmacol.*, **60**, 221-231

PARKMAN, H.P., TRATE, D.M., KNIGHT, L.C., BROWN, K.L., MAURER, A.H. & FISHER, R.S. (1999). Cholinergic effects on human gastric motility. *Gut*, **45**, 346-354

PRINS, N.H., AKKERMANS, L.M.A., LEFEBVRE, R.A. & SCHUURKES, J.A.J. (2000a). 5-HT_4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br. J. Pharmacol.*, **131**, 927-932

PRINS, N.H., SHANKLEY, N.P., WELSH, N.J., BRIEJER, M.R., LEFEBVRE, R.A., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (2000b). An improved *in vitro* bioassay for the study of 5-HT_4 receptors in the human isolated large intestinal circular muscle. *Br. J. Pharmacol.*, **129**, 1601-1608

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 864-989

TONINI, M., DE GIORGIO, R., DE PONTI, F., STERNINI, C., SPELTA, V., DIONIGI, P., BARBARA, G., STANGHELLINI, V. & CORINALDESI, R. (2000). Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations. *Br. J. Pharmacol.*, **129**, 12-20

CHAPTER 1

LITERATURE SURVEY

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1.1. Innervation of the gastrointestinal tract

1.1.1. General

In humans, the gastrointestinal (GI) tract consists of a 6-9 m long canal, beginning at the mouth and ending at the anus, together with the associated organs that empty their contents in the tract. The GI tract consists of the mouth, the oesophagus, the stomach, the small intestine (divided in the duodenum, jejunum and ileum), and the large intestine (divided in the colon and rectum). The major physiological processes that occur in the GI tract are secretion, digestion, absorption, motility and elimination. Food enters the mouth where it will be reduced in size before moving to the stomach *via* the oesophagus. The stomach will store the food, mix it with its secretions containing pepsin, ions and water, and grind it until the particles are small enough to pass the pylorus and enter the small intestine. In the small intestine, enzymes convert the macromolecules into absorbable material, a process called digestion; the products of the digestion will be absorbed across the epithelium of the small intestine to enter the blood or lymph. Finally, undigested food will reach the colon where water will be resorbed before the contents leave the body. During the grinding and mixing, motility is important, which is mediated by the smooth muscle layer of the GI tract. Motility is also necessary for the progression of the contents throughout the tract and for eliminating the undigested food *via* the faeces.

Although the GI tract consists of different regions, the structure of all these regions is quite similar and consists of four layers (Figure 1.1): the inner layer is called the mucosal layer and can be divided into the mucosa and the mucosal muscle layer; the second layer is the submucosa with the submucosal neuronal plexus or plexus of Meissner; the third layer consists of the circular and longitudinal muscle layers and in between the myenteric neuronal plexus or plexus of Auerbach. Finally, the serosa forms the outer layer that surrounds the tract.

The two plexuses control the multiple functions of the GI tract, such as secretion, absorption and propulsion, and are known as the enteric nervous system (ENS). Besides this intrinsic control, the GI tract also receives extrinsic input from the central nervous system

(CNS) *via* parasympathetic and sympathetic neurones. These two control levels are described more in detail in the next two sections, and the following reviews concerning the innervation of the GI tract form the basis for these sections (Costa & Brookes, 1994; Furness *et al.*, 1996; Goyal & Hirano, 1996; Bennett, 1997; Kunze & Furness, 1999).

1.1.2. Extrinsic innervation

The parasympathetic and sympathetic nervous system both contain efferent and afferent fibers. Both efferent systems have a bi-neural connection with the effector cells. The preganglionic nerves in both systems are cholinergic, releasing the neurotransmitter acetylcholine.

The parasympathetic nervous system towards the GI tract consists of two nerves: 1) the vagal nerve, originating in the medulla oblongata and innervating the major part of the GI tract as far as the mid-transverse colon, and 2) the pelvic nerve, originating from the sacral cord and innervating the lower part of the colon (Figure 1.2). About 80-90 % of the parasympathetic neurones are afferent, and transmit information from the sensory nerve endings in the gut through their cell bodies in the nodose ganglia to the CNS. Sensory nerve

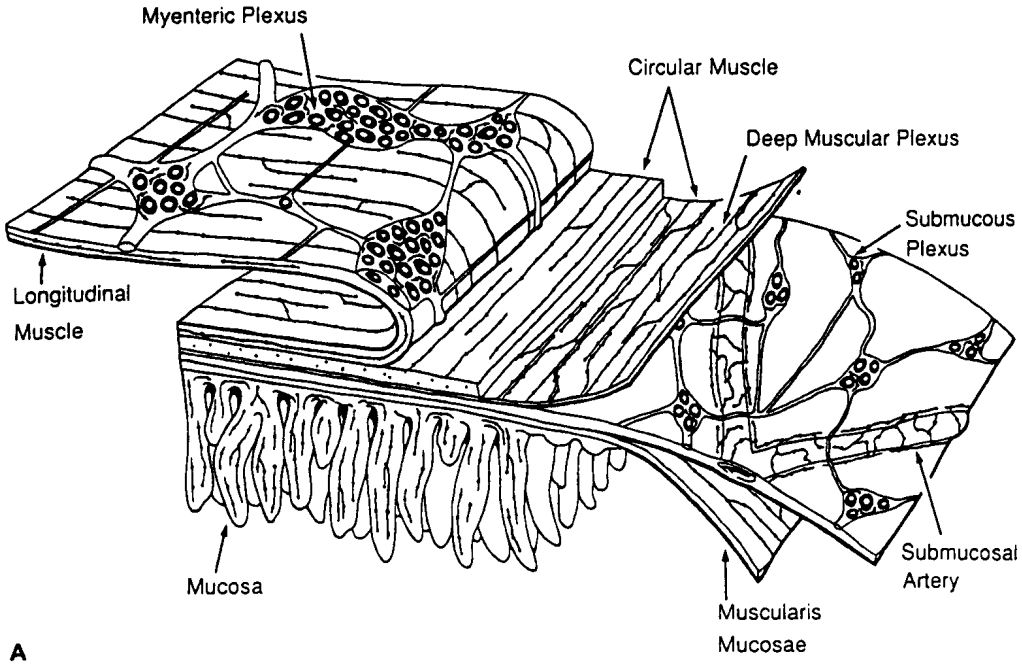

Figure 1.1 Schematic representation of the different layers of the gastrointestinal tract, and the location of the enteric plexuses in whole mounts of intestine. The serosa is not shown. (Adapted from Furness & Costa, 1987).

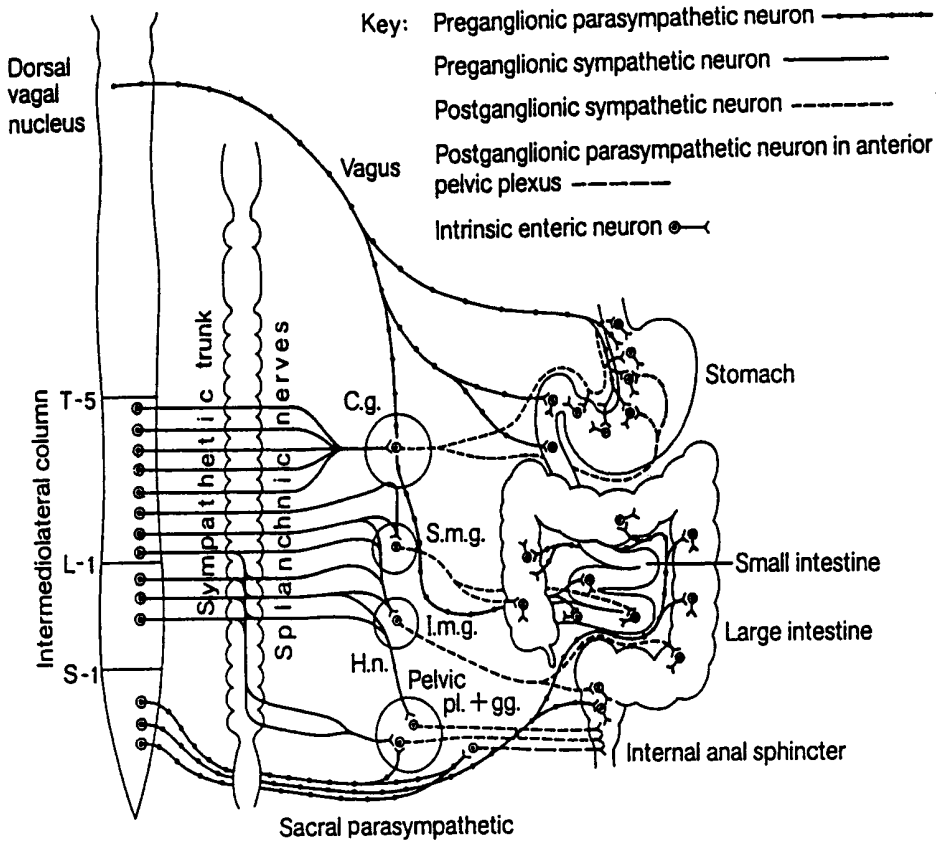

Figure 1.2 The extrinsic efferent innervation of the gastrointestinal tract. C.g.: celiac ganglion, S.m.g.: superior mesenteric ganglion, I.m.g.: inferior mesenteric ganglion, Pelvic pl. + gg.: pelvic plexus and ganglia, H.n.: hypogastric nerve. (Adapted from Baumgarten, 1982).

endings in the mucosa are sensitive to luminal concentrations of glucose, amino acids or long-chain fatty acids, others respond to a wide variety of transmitters and mechanical stimuli; sensory nerve endings in the muscle layers are sensitive to mechanical distension of the gut. In the CNS, the sensory neurones connect to the efferent vagal neurones. Only 10-20 % of the parasympathetic fibers are efferent, innervating the GI tract. These efferent fibres release acetylcholine, and stimulation of these fibers generally activates nicotinic receptors within the intrinsic enteric ganglia of the gut. The classic parasympathetic fibers synapse in this way with excitatory cholinergic neurones in the gut wall. However, it has become clear that vagal preganglionic fibers also synapse with non-adrenergic non-cholinergic (NANC) neurones.

The processes of the efferent sympathetic nervous system, originating in the thoraco-lumbar part of the spinal cord, run through the paravertebral ganglia, and the preganglionic neurones release acetylcholine, activating nicotinic receptors on the neuronal cell bodies in the prevertebral ganglia (celiac, superior and inferior mesenteric) (Figure 1.2). From these

cell bodies, postganglionic noradrenergic fibers project to the enteric ganglia *via* the splanchnic nerves, having at least four distinct targets in the gut: 1) they influence secretomotor neurones containing vasoactive intestinal polypeptide (VIP); 2) they affect the blood supply to the musculature but do not directly innervate the smooth muscle cells, 3) except for most sphincter regions where they contract the smooth muscle by a direct effect on the muscle; and 4) they reduce GI motility in non-sphincteric regions primarily through actions of the noradrenergic axons in the myenteric ganglia, inhibiting the release of excitatory neurotransmitters from local nerve terminals e.g. inhibition of acetylcholine release from excitatory cholinergic neurones *via* presynaptic α_2-adrenoceptors on the cholinergic nerve endings (see review De Ponti *et al.*, 1996). Besides this efferent limb, there is also transfer of sensory information to the CNS by neurones having their endings in the gut wall and their cell bodies in the dorsal root ganglia. These neurones are nociceptors, sensing high-intensity mechanical, thermal and chemical stimuli that damage or threaten the tissue.

1.1.3. Intrinsic innervation

The ENS is a collection of about 100 million neurones in the GI tract that constitutes the "brain of the gut" and can function independently of the CNS, although it communicates with the CNS through sympathetic and parasympathetic afferent and efferent neurones (see above). The ENS controls the motility, exocrine and endocrine secretions, and microcirculation of the GI tract; it is also involved in regulating immune and inflammatory processes.

In the ENS, the nerve-cell bodies are grouped into small ganglia, that are connected by bundles of nerve processes forming two major neuronal plexuses, called the myenteric or Auerbach's plexus and the submucosal or Meissner's plexus (Figure 1.1). The myenteric plexus extends over the entire length of the gut, regulating GI motility. The submucosal plexus is best developed in the small intestine, where it plays an important part in the control of secretion and absorption, although it also innervates the muscularis mucosae, intestinal endocrine cells and submucosal blood vessels. The ganglia and connecting nerve bundles are surrounded by a basal lamina, collagen fibrils and enteric glial cells. Smaller plexuses are also found such as the deep muscular plexus, lying deep within the circular muscle layer of the intestine (Figure 1.1).

Four functional types of enteric neurones are described in the ENS: the secretory and motor neurones, both having efferent functions, the sensory neurones, also called the

'intrinsic primary afferent neurones' (IPANs), and the interneurones. The motor neurones, IPANS and interneurones are involved in reflexes, including peristalsis, an important reflex for the motility in the small and large intestine. These neurones are connected with each other and form a complex system, the ENS. The sensory endings of the IPANs are present in the mucosa, with their cell bodies in the myenteric plexus. They are sensitive to different stimuli like distension, changes in luminal content and 5-hydroxytryptamine (5-HT), and they will send information to the myenteric plexus. Some of the IPANs have their cell bodies in the submucosal plexus; stimulation of these IPANs might be involved in controlling the transmucosal water and electrolyte transport and local blood flow. In the myenteric plexus the IPANs form connections with longitudinally projecting interneurones, the latter running orally or anally and designated as ascending or descending respectively (Figure 1.3). They form multisynaptic pathways and make connections with both motor neurones and other interneurones; the latter can explain why enteric reflexes often extend for several centimetres along the intestine from a single point of stimulus. Ascending interneurones contain acetylcholine, acting on nicotinic receptors of other interneurones and motor neurones; however, the transmitters in the descending interneurones are not yet known. Finally, the motor neurones project to the muscle layers (Figure 1.3). These neurones are either excitatory or inhibitory. Excitatory motor neurones cause contractions of the muscles orally of their cell bodies due to the release of acetylcholine and probably substance P. On the other hand, inhibitory motor neurones cause relaxations of the muscles anally to their cell bodies due to the release of nitric oxide (NO), VIP and other inhibitory neurotransmitters; these neurotransmitters can be released from the same neuron. Neurotransmitters might interfere with each others release and action. The axons run circumferentially, following the direction of the muscle cells, and as the cells are electrically coupled to each other they influence the muscle cells as a group.

Information from both the excitatory and inhibitory motor neurones is transferred to the smooth muscle at least in part *via* the interstitial cells of Cajal (ICC), which are electrically coupled to the muscle. ICCs are non-neural cells that serve as pacemakers and are responsible for the spontaneous, rhythmic, electrical excitatory activity of GI smooth muscle that is referred to as slow waves. The ICCs are susceptible to both the inhibitory transmitter NO, and the excitatory tachykinin transmitters.

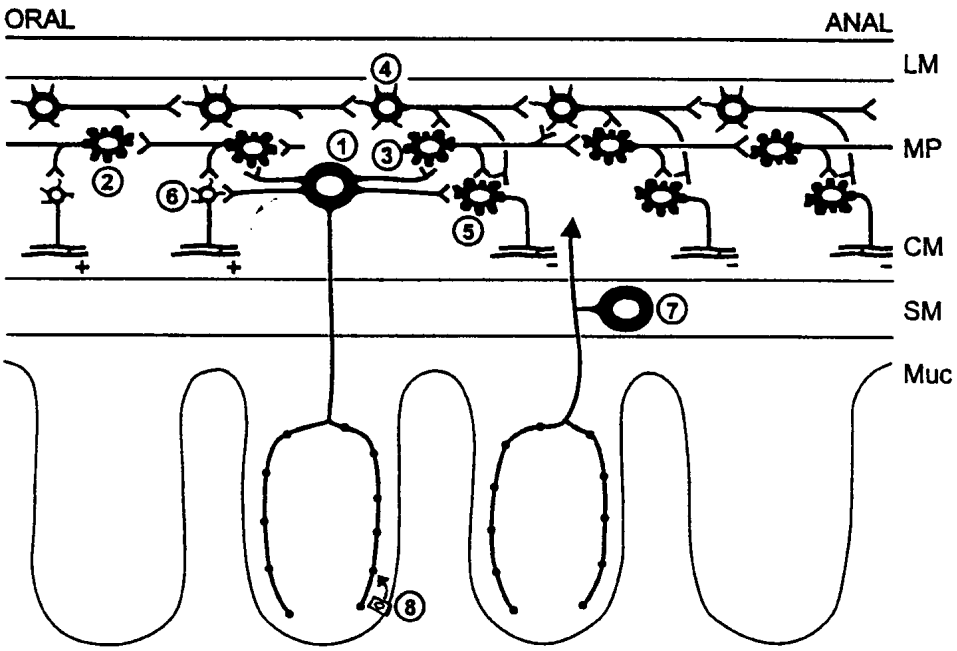

Figure 1.3 Simplified, in series representation of enteric circuits for motility reflexes. 1: IPAN with cell body in the myenteric plexus; 2: ascending cholinergic interneuron; 3: descending interneuron in the local reflex pathway; 4: descending interneuron of the migrating motor complex pathway; 5: inhibitory motor neuron; 6: excitatory motor neuron; 7: IPAN with cell body in the submucosal plexus; 8: enteroendocrine cell that releases an excitant of the mucosal endings of IPANs; LM: longitudinal muscle; MP: myenteric plexus; CM: circular muscle; SM: submucosa; Muc: mucosa. (Adapted drom Kunze & Furness, 1999).

1.1.3.a. Cholinergic innervation

Cholinergic neurones are found all over the body. These neurones release the neurotransmitter acetylcholine. Choline, taken up by the cholinergic neurones, is linked to acetyl-CoA by the enzyme choline acetyltransferase to form acetylcholine, which will be taken up into vesicles until it is released after activation of the neurones. This acetylcholine will have its effect by activation of two different types of receptors: nicotinic and muscarinic receptors; the effect of acetylcholine at these receptors is indeed mimicked by nicotine and muscarine respectively. To end the effect of acetylcholine, acetylcholine will be broken down into acetate and choline by the enzyme acetylcholinesterase. Choline will be recovered by the cholinergic neurones.

The preganglionic neurones of the parasympathetic and sympathetic nervous system, as mentioned before, and the neurones innervating the skeletal muscle cells release acetylcholine. This acetylcholine will activate nicotinic receptors present at the somata of postganglionic neurones and at the end plate of skeletal muscle cells. These receptors are

built up by five subunits (two α, and one β, γ and δ), around a channel. After each α-subunit is occupied by acetylcholine, the central pore opens, resulting in an increased permeability for sodium and potassium ions, and consequently a depolarisation of the effector cell. The nicotinic receptors on the postganglionic neurones can be blocked by hexamethonium.

Acetylcholine released from cholinergic neurones ending on effector cells interacts with muscarinic receptors. They are amongst others localized on neuronal cell bodies in the CNS, on cardiac muscle, on smooth muscle cells and on exocrine glands. Five different muscarinic receptor subtypes have been identified based on studies of molecular structure, *in vitro* binding and function (Buckley *et al.*, 1989; Dörje *et al.*, 1991). All subtypes belong to the seven-transmembrane G-protein coupled receptor family (see reviews Grimm *et al.*, 1994; Eglen *et al.*, 1996; Caulfield & Birdsall, 1998). Muscarinic M_1, M_3 and M_5 receptors preferentially couple to a membrane-bound phospholipase C *via* stimulatory $G_{q/11}$-protein, resulting in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes release of calcium from intracellular stores, while DAG activates protein kinases (PK), especially PKC and is metabolised to arachidonic acid and derivatives. The M_2 and M_4 receptors preferentially inhibit adenylyl cyclase *via* activation of inhibitory $G_{i/o}$-proteins. Other minor pathways might also be involved. The muscarinic receptors can be blocked by the non-selective antagonist atropine; more selective muscarinic antagonists are also known.

In the GI tract, the ascending motor neurones and interneurones, as well as most intrinsic sensory neurones are cholinergic, but they contain very often, apart from acetylcholine, also tachykinins. These neurones project to the smooth muscle cells and to other neurones, and the released acetylcholine stimulates muscarinic receptors, resulting in contraction. At the postsynaptic level, M_2 and M_3 receptors are present, and although the majority of the receptors belong to the M_2 subtype, mainly the M_3 receptors are detected pharmacologically (Eglen *et al.*, 1996; Caulfield & Birdsall, 1998). M_2 receptors become more important after stimulation of adenylyl cyclase (Ehlert *et al.*, 1999), or during inflammation (Shi & Sarna, 1997, 1999). The released acetylcholine can also interfere at the presynaptic level with its own release and the release of other neurotransmitters. However, the situation at this level is more complex as both stimulatory and inhibitory muscarinic receptors can be present (Grimm *et al.*, 1994).

1.1.3.b. Nitrergic innervation

For many years it was believed that acetylcholine and noradrenaline were the only two neurotransmitters in the GI tract. However, since the 1960's it became evident that other neurotransmitters exist, which were indicated as non-adrenergic non-cholinergic (NANC; see review Bennett, 1997). The NANC neurones now represent a large class of neurones which can contain contractile neurotransmitters like substance P (Shuttleworth & Keef, 1995), or inhibitory neurotransmitters like adenosine triphosphate (ATP; Burnstock, 1972), vasoactive intestinal polypeptide (VIP; Fahrenkrug, 1982) or nitric oxide (NO; Sanders & Ward, 1992).

Inhibitory NANC neurones are recognized in the urogenital, respiratory, cardiovascular and GI tract. The functional significance of the inhibitory NANC neurones in the GI tract is important in many physiological and pathophysiological conditions (Goyal & Hirano, 1996). An important portion of the inhibitory NANC neurones in the GI tract use NO as their neurotransmitter.

NO is a small labile molecule, with a half-life of 3 to 50 s. It is enzymatically formed together with L-citrulline from the amino acid L-arginine (Figure 1.4) by the catalytic activity of a family of iso-enzymes, the NO synthases (NOS). Three iso-enzymes of NOS, encoded by three different genes, have been identified: neuronal NOS (nNOS, bNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). These isoforms vary in tissue distribution, transcriptional regulation, cofactor requirements, posttranslational modification and function. All three isoforms need flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and protoporphyrin IX haem as well as two co-substrates (O_2 and NADPH) (Schmidt *et al.*, 1993). L-arginine binds to NOS, resulting in the oxidation of L-arginine whereby the intermediary L-N^G-hydroxyarginine is formed, that is further oxidized to form NO and its co-product L-citrulline. All NOS isoforms are inactive until calmodulin binds to them in a process which can be either calcium-dependent (eNOS and nNOS) or calcium-independent (iNOS). nNOS and eNOS are constitutively expressed, calmodulin-free and inactive. An increase of the amount of free intracellular calcium allows that these two NOS isoforms bind calmodulin and become active. nNOS is mostly found in neurones, and the produced NO regulates many functions in the CNS; in the peripheral nervous system, NO plays an important role as inhibitory NANC neurotransmitter in the GI, respiratory and urogenital tract (Toda *et al.*, 2000). eNOS was first identified in endothelial cells (Pollock *et al.*, 1991), and contributes to the regulation of the blood pressure. iNOS on the other hand is mainly expressed after activation of cells such

as macrophages with different cytokines or endotoxins. iNOS binds calmodulin constitutively and is thus constitutively active, independently of the intracellular Ca^{2+} concentration. The NOS isoforms can be inhibited by L-arginine analogues that stereotypically inactivate NOS such as N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME).

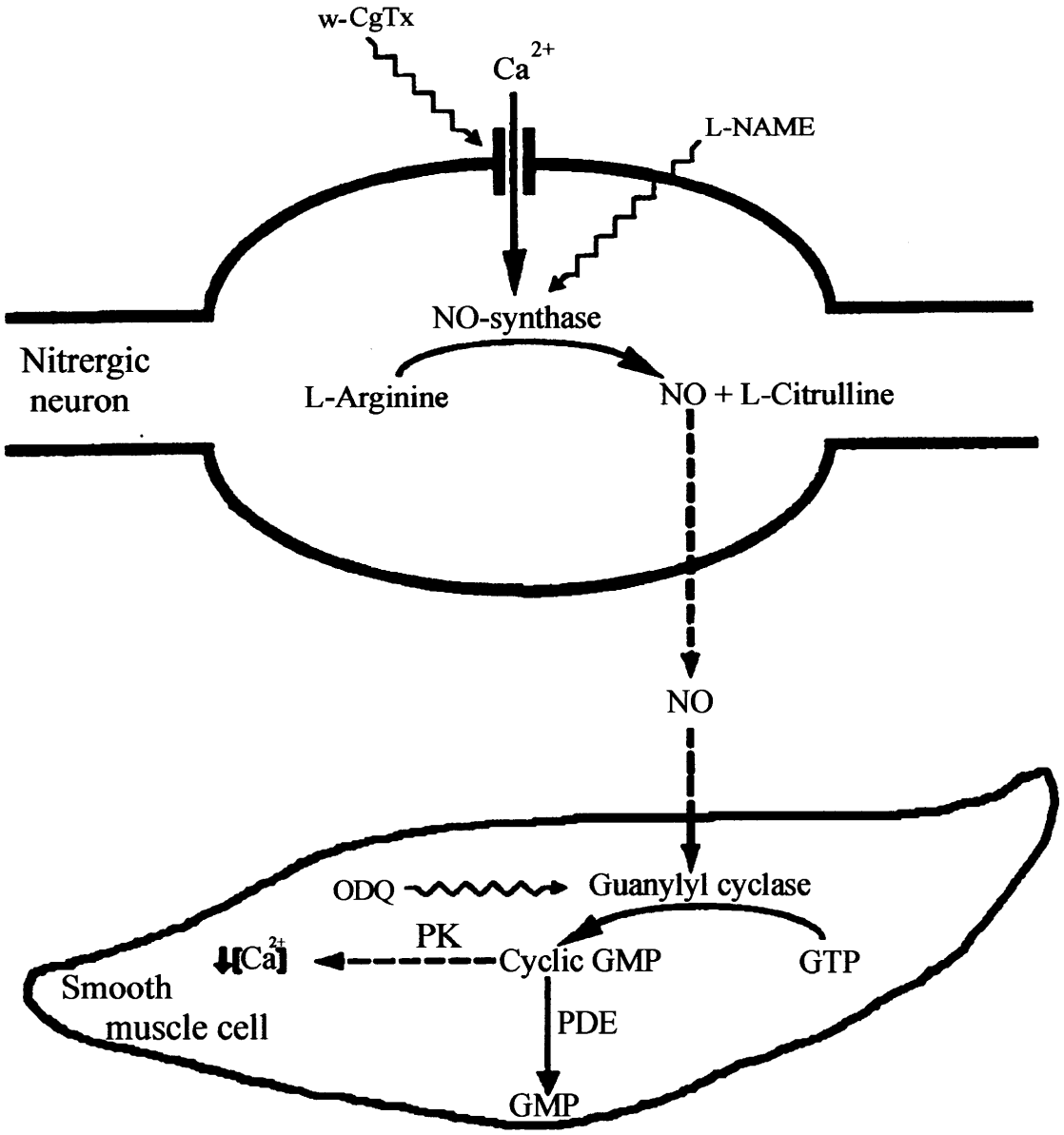

Figure 1.4 Schematic representation of nitrergic neurotransmission. Upon an action potential, N-type calcium channels open, allowing calcium to enter the neuron. The increase in intracellular calcium activates NO synthase, resulting in production of NO. NO diffuses into the extracellular space and into nearby effector cells such as smooth muscle cells. NO activates soluble guanylyl cyclase in these cells, resulting in an increase of cyclic GMP. This cyclic GMP activates protein kinase (PK), resulting in a decrease in the intracellular calcium concentration and muscle relaxation. Phosphodiesterases (PDE) stop the relaxation by breaking down cyclic GMP. The transmission process can be blocked by inhibiting the neuronal calcium channels with ω-conotoxin (w-CgTx), by inhibiting NO synthase with e.g. L-NAME, or by inhibiting guanylyl cyclase with ODQ.

The lipophilic molecule NO diffuses outwards into nearby target cells where it binds to the haem prosthetic group of soluble guanylyl cyclase and stimulates the intracellular accumulation of guanosine 3'5' cyclic monophosphate (cyclic GMP) from guanosine 5' triphosphate (GTP) (Figure 1.4). Depending on the cell and tissue type, cyclic GMP regulates cyclic GMP-dependent protein kinase, cyclic GMP-regulated phosphodiesterase and cyclic GMP-gated ion channels. Bolotina *et al.* (1994) also reported a role for NO as vasorelaxant independently of cyclic GMP by activating directly Ca^{2+}-dependent potassium channels causing hyperpolarisation. When NO is formed in large quantities by iNOS, it can also act via other signal transduction pathways than described above, often resulting in a loss of function of the cell.

The main inhibitory neurotransmitter in the GI tract is NO. In the stomach it is involved in the receptive as well as the adaptive relaxation; in the small and large intestine, it has been demonstrated that the anally orientated motor neurones contain NO, causing relaxation of the gut descending of a propagating bolus. Although NO is an important neurotransmitter in the GI tract, immunohistochemical studies demonstrated that VIP is often co-localized with NO in the myenteric plexus innervating the GI tract (Furness *et al.*, 1992; Lefebvre *et al.*, 1995; Tonini *et al.*, 2000), and contractility studies demonstrated that this VIP plays a role in NANC relaxations. However, this does not mean that both transmitters are always released together and contribute to the relaxations in a similar extent. In rat, ferret and man stomach, NO initiates and VIP sustains the relaxation (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; Grundy *et al.*, 1993; Tonini *et al.*, 2000); in guinea-pig, cat and pig gastric fundus, NO is the main inhibitory NANC transmitter (Lefebvre *et al.*, 1992a, 1995; Barbier & Lefebvre, 1993; Desai *et al.*, 1994). Binding of VIP with its receptor stimulates G-proteins that activates adenylyl cyclase, resulting in an increased production of adenosine 3'5' cyclic monophosphate (cyclic AMP) from adenosine 5' triphosphate (ATP), and consequently relaxation of the smooth muscle.

1.2. Gastrointestinal motility

This study concentrates on the motility of the stomach and colon. For this reason, the motility of these parts of the GI tract are described more in depth in the next two sections.

1.2.1. Stomach

The stomach is a saclike structure, located in the upper abdomen just below the diaphragm; it contains the four layers as described in section 1.1.1. The stomach starts the breakdown of an incoming meal by its secretory and motor responses. The stomach secretes about 3 l of gastric juice daily. This fluid consists of water, hydrochloric acid, intrinsic factor, pepsin and mucin, the main component of mucus which protects the stomach from the effects of the acid and pepsin. Hydrochloric acid creates the acid environment necessary for pepsin to begin the breakdown of proteins, and it destroys most of the ingested micro-organisms. The motor functions of the stomach include acting as a reservoir for ingested food, grinding the food and emptying of the contents into the duodenum at controlled rates. When considering these motor functions, the stomach can be divided into two parts: the proximal and distal part (Figure 1.5). The proximal stomach consists of the fundus and oral one-third of the corpus; it shows tonic motility changes and is thought to function mainly as a reservoir for the storage of liquids and solids. It also controls the emptying of liquids into the small intestine. The distal part consists of the remaining corpus, the antrum and the pylorus; it shows phasic contractility and is involved in grinding the particles and controlling the emptying of solids. Coordinated actions of these regions with feedback control from the small and large intestine regulate emptying of the gastric contents into the duodenum (Kelly, 1980; Hasler, 1996a).

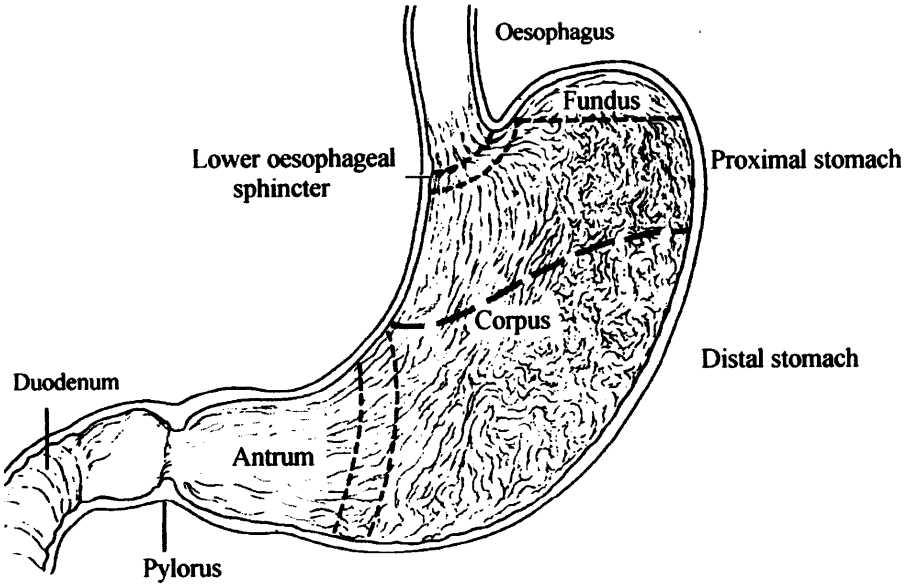

Figure 1.5 Schematic representation of the different parts of the stomach.

The proximal stomach regulates intragastric pressure during gastric filling due to its relaxant properties. During swallowing, the pharynx and oesophagus are distended, activating mechanoreceptors. The information of the mechanoreceptors is transported to the brain stem by afferent neurones, which will activate efferent vagal preganglionic fibers, that synapse with inhibitory NANC neurones in the gastric wall. This vago-vagal reflex pathway, activating NANC neurones, causes relaxation of the proximal part of the stomach which allows the proximal stomach to receive the food with minimal increases in pressure; this is called the receptive relaxation (Abrahamsson, 1973, 1986) (Figure 1.6). When the intake of a meal is finished, the oesophago-gastric reflex of the stomach stops as well. Instead, the distension of the stomach, or the localised distension of the distal stomach by the gastric contents activates distension receptors, which elicit vago-vagal reflex responses maintaining the relaxation of the proximal stomach *via* inhibitory NANC neurones, being responsible for the long term relaxation, the adaptive relaxation (Abrahamsson, 1973, 1986) (Figure 1.6). Besides of efferent fibers ending on inhibitory NANC neurones in the gastric wall, the vagus also contains efferent preganglionic fibers synapsing with contractile cholinergic neurones in the gastric wall. However, the vagal efferent supply in the reflex pathway to the stomach seems organised in a reciprocal manner at the central level: when the vagal preganglionic efferents supplying the intrinsic inhibitory NANC neurones are active as is the case during intake of a meal, the discharge in those supplying the intrinsic excitatory cholinergic neurones is suppressed; during fasting the opposite is seen (Andrews, 1990). At the level of the stomach, evidence has accumulated that both NO and VIP are involved in NANC relaxation (Lefebvre, 1993).

Mixing and grinding of the gastric content, as well as emptying of the stomach, especially of solids, are regulated by the distal stomach. In the upper part of the distal stomach at the greater curvature, a pacemaker generates slow waves. These slow waves, consisting of an initial rapid depolarisation followed by a more prolonged plateau potential, propagate in the direction of the small intestine (Hasler, 1996a). Another pacemaker is present in the duodenum. Under quiescent conditions, the slow waves are not of sufficient amplitude to reach the threshold to induce a significant contraction in the distal stomach. However, these slow waves can be modified: intake of food causes distension of the proximal stomach, activating a vagal fundo-antral reflex pathway with stimulation of cholinergic neurones. These neurones increase the amplitude of the slow waves whereby the plateau potential of the depolarisation is prolonged and enhanced so that the threshold to initiate an action potential is reached, resulting in circular contractions that propagate over the corpus

and antrum towards the pylorus. These contractions are of variable intensity and duration, and solid food induces stronger antral contractions than a similar load of liquids. The contractions propel the gastric content distally. However, while the ingested food moves to the distal antrum, the pylorus closes due to the activation of cholinergic neurones, resulting in retropulsion of the mixed bolus into the proximal stomach. These actions serve to grind and mix the luminal contents (Kelly, 1980; Hasler, 1996a). The movements reduce the food into particles small enough to pass the pylorus when the sphincter is open due to activation of nitrergic neurones during the short moments just before the antral contractions reach the pylorus and the pylorus closes.

In addition to these reflexes which occur during the fed period, the GI tract exhibits between two meals cyclic changes of activity that pass along the tract from the stomach to the terminal ileum. These interdigestive patterns, known as migrating motor complexes (MMC),

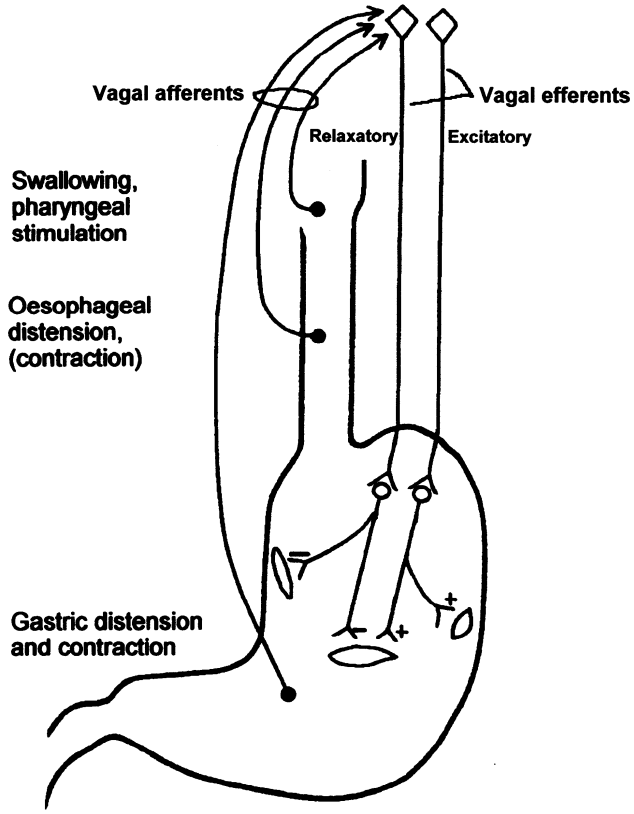

Figure 1.6 Schematic representation of the vagal innervation of the smooth muscle cells in the proximal stomach. Shown are the reflex mechanisms producing gastric receptive and adaptive relaxation *via* the vagal non-adrenergic relaxatory fibres to the stomach.

clear the stomach of undigested debris and sloughed epithelial cells. The MMC consists of three phases: phase I is a period of motor quiescence, which is followed by a phase II with increasing but irregular contractions. Phase III is a period of intense, rhythmic, luminally occlusive contractions that mostly begin in the corpus at the pacemaker and propagate unimpeded to the pylorus, although it sometimes even starts in the distal duodenum. These contractions are highly propagative. During phase III, the pylorus is open to allow easy clearing of the stomach. There is evidence that the gastric phase III activity is induced by motilin, while the extrinsic innervation only modulates MMC activity. Sometimes a phase IV is described, a brief period of less intense activity from phase III to phase I. This interdigestive pattern disappears soon after a meal is taken, to be replaced by the fed pattern activity described above (Hasler, 1996a).

During both the fed and interdigestive periods, a entero-gastric reflex pathway modulates the emptying of the stomach, mostly resulting in a delay in gastric emptying or a prolonged MMC.

1.2.2. Colon

In humans, ingesta from the small intestine enter the large intestine through the ileocaecal junction, a sphincter preventing the reflux of material from the colon to the terminal ileum. The large intestine consists of the caecum, colon and rectum. The caecum is the first part that receives the food particles. The colon, 91 – 125 cm long, can be divided into four regions: the ascending, transverse, descending and sigmoid colon (Figure 1.7 A.). The caecum, ascending and transverse colon and the rectosigmoid area act as a reservoir, while the descending colon acts as a conduit. Although the same layers are present in the colon as in the other regions of the GI tract, the organisation is slightly different: the colon possesses a circumferential circular muscle layer with on top three thin layers of longitudinal muscle, the taenia coli (Figure 1.7 B). Contractions of the circular muscle that narrow the lumen, together with longitudinal contractions that shorten the length of the colon give rise to colonic haustra (Figure 1.7 A and B). The rectum, 15 – 20 cm long, contains circumferential longitudinal and circular muscle layers as in the other regions of the GI tract. The major functions of the colon are extraction of water, digestion of certain meal residues and processing of faeces for controlled expulsion. The large intestine contains, in contrast to the other parts of the GI tract, large amounts of bacteria, that metabolise unabsorbed nutrients in the ascending and

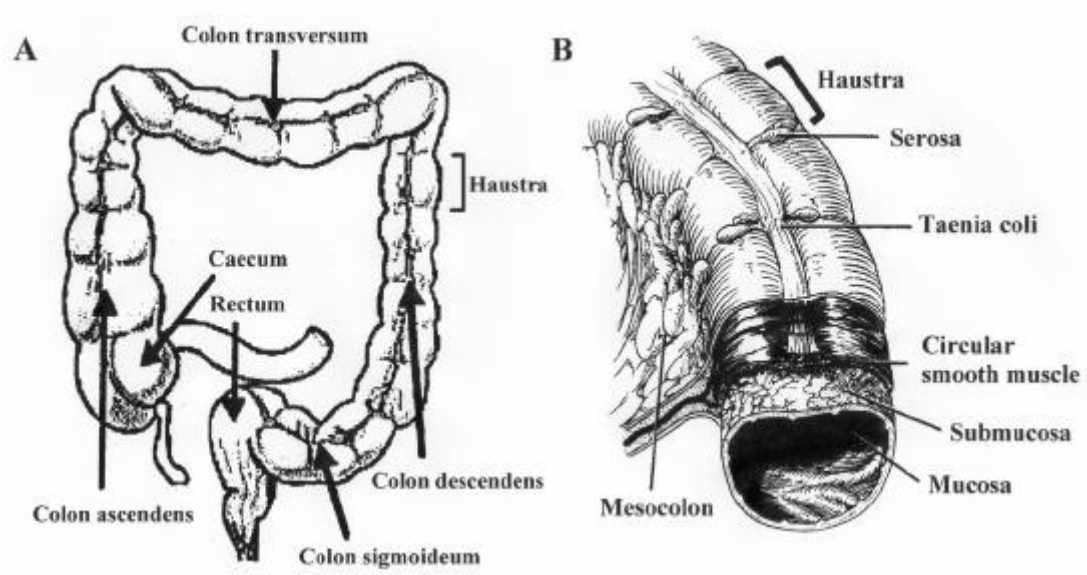

Figure 1.7 **A.** Schematic representation of the different regions of the large intestine. **B.** Schematic representation of the different layers of the colon. The enteric plexuses are not shown. (Adapted from Junqueira *et al.*, 1993).

transverse colon. The bacteria also produce vitamin K, a crucial factor for coagulation. These products will be absorbed by the colon, together with about 6 l water daily to make the stool solid. To recover that large amount of water, the faecal material may not propagate to fast to allow enough time for absorption. Indeed, if stool moves too quickly, not enough water is absorbed, resulting in diarrhea; however, when the motility of the colon is too low, the colon can absorb too much water, resulting in hard stool and constipation (Camilleri & Ford, 1998). Colonic motility must thus be well regulated by the combined actions of several distinct motor patterns and their corresponding myoelectrical complexes.

The circular muscle layer is the most important one with regard to colonic motility. Three different motor patterns can be recognized in the colon: local contractions, peristaltic reflexes and giant migrating contractions (GMCs).

Local contractions, originating from pacemakers in the submucosal and myenteric border located throughout the whole length of the colon, will mix the faecal material to promote extraction of water; the faeces hereby only propagate for short distances in an oral or aboral direction.

At the same time, peristaltic reflexes press the faecal material aborally to the left side of the colon, particularly to the rectosigmoid, to store it before it will be removed. The peristaltic reflex originates from IPANs, with their cell bodies in the myenteric plexus, that are activated by distension and changes in luminal content. These neurones activate

interneurones and ascending contractile cholinergic neurones and descending inhibitory NANC neurones. However, a retroperistaltic transit pattern from the pacemaker area in the transverse colon into the ascending colon has been observed, slowing down the propagation of the faecal matter and increasing the absorption. This results in a transit of one to two days. Faeces are stored for the longest period in the rectosigmoid, supporting its role as a reservoir. Rectal motor complexes facilitate the storage of the colonic content that reaches the rectum, probably by pressing the content together. These contractions occur about 16 times a day, and do not propagate. During these complexes, no anal relaxations are observed, consistent with a role in maintenance of faecal continence. In contrast to the upper GI tract, there is no organised interdigestive, cyclical MMC in the human colon (Hasler, 1996b).

Ingesta from the small intestine as well as the slow propagation of the faecal contents causes a large distension of the proximal colon, inducing GMCs, which propagate aborally from the proximal colon to the distal colon and evoke mass movements of faeces. These GMCs are generated by myenteric pacemakers, and thus propagate in motor patterns distinct from the peristaltic reflex (Hasler, 1996b). During the first three to four years of life, the number of GMCs progressively decreases from three or four per day until an average of two GMCs per day is reached, which remains constant until the frequency in the elderly population decreases significantly (Hillemeier, 1995). Meal ingestion is an important stimulus to increase motor activity in the colon *via* the cholinergic neural pathway of a gastrocolonic response. This is sometimes followed by mass movements and defecation. No GMCs occur during periods of sleep, while the other colonic motor activities are infrequent. However, motor activity increases soon after awakening, often leading to defecation.

The GMCs bring an important mass of faeces into the rectum, resulting in a sudden distension of the rectum. Mechanosensitive enteric neurones register the distension and activate a rectoanal inhibitory reflex (Hillemeier, 1995; Hasler, 1996b). The reflex decreases the pressure of the internal anal sphincter due to stimulation of inhibitory NANC neurones with release of NO and VIP. Normally the pressure of this sphincter is sufficient to prevent accidental loss of faecal material. However, the pressure of the external anal sphincter increases with the distension of the rectum, preventing that the faeces can leave the body after the internal anal sphincter opens, unless it is convenient. The mechanoreceptors also transmit the message to the CNS to register that it is time for defecation. In order to allow defecation, the individual must voluntarily relax the external anal sphincter and associated skeletal muscle structures and increase abdominal pressure.

1.3. Presynaptic modulation of cholinergic neurotransmission

Under quiescent conditions and certainly upon stimulation, cholinergic neurones release acetylcholine acting at the postsynaptic level on muscarinic or nicotinic receptors. The released acetylcholine can also interact at the presynaptic level whereby acetylcholine modifies its own release *via* autoreceptors or the release of other neurotransmitters *via* heteroreceptors. On the other hand, other neurotransmitters may facilitate or inhibit the release of acetylcholine, mostly *via* stimulation of receptors on the cholinergic nerve endings; NO is able to influence the release of acetylcholine without binding on a receptor. This section focuses on the modulation of acetylcholine release *via* autoreceptors and heteroreceptors on cholinergic nerve endings modulating GI motility.

1.3.1. Presynaptic modulation via muscarinic receptors

Although a lot of evidence exists that muscarinic receptors are present on the cholinergic nerve endings innervating the GI smooth muscle, regulating the release of acetylcholine, mainly *via* inhibition, few studies characterised the subtype of presynaptic muscarinic autoreceptor(s). This is probably due to the lack of very selective muscarinic agonists and antagonists. For this reason, a series of subtype-preferring muscarinic receptor antagonists have to be tested, and their affinity at the muscarinic receptors have to be compared with affinity values obtained from binding studies for the muscarinic receptor subtypes. Muscarinic subtype preferring antagonists exist (Eglen *et al.*, 1996; Caulfield & Birdsall, 1998) such as the non-selective antagonist atropine, the M_1-preferring antagonist pirenzepine, the M_2-preferring antagonists AF-DX 116 and methoctramine, the M_3-preferring antagonists 4-DAMP and p-F-HHSiD, and the very selective M_4-receptor antagonist MT-3 (Adem & Karlsson, 1997). In guinea-pig longitudinal muscle-myenteric plexus (LMMP) preparations of the ileum, presynaptic muscarinic M_3 receptors inhibit while presynaptic M_1 receptors enhance acetylcholine release (Soejima *et al.*, 1993). On the contrary, presynaptic inhibitory muscarinic M_1 receptors are present on the cholinergic nerve endings innervating the circular smooth muscle of the guinea-pig ileum (Dietrich & Kilbinger, 1995), indicating that in the same region of a given species, both stimulatory and inhibitory muscarinic M_1 receptors can be present depending on the muscle layer studied. In the guinea-pig stomach, it has been demonstrated that presynaptic muscarinic M_1 and M_2 receptors inhibit [^{3}H]-

acetylcholine release (Ogishima *et al.*, 2000). In canine LMMP preparations, a binding study demonstrated the presence of presynaptic M_3 receptors, although the presence of another presynaptic muscarinic subtype could not be excluded (Kostka *et al.*, 1989).

1.3.2. Presynaptic modulation via α_2-adrenoceptors

As mentioned before, the noradrenergic neurones of the sympathetic nervous system generally do not innervate the smooth muscle cells directly, but have their effect indirectly by influencing the release of other neurotransmitters, including the release of acetylcholine. Two main adrenergic receptor types have been identified, and these are further divided into subclasses, leading to α_1-, α_2-, β_1-, β_2- and β_3-adrenoceptors. Selective adrenoceptor agonists and antagonists are used to study the effect of the sympathetic nervous system on the release of acetylcholine, and which type of adrenoceptor is involved. Phentolamine, a selective α-adrenoceptor antagonist, is used to demonstrated the presence of α-adrenoceptors. However, more selective α_2-adrenoceptor agonists and antagonists exist nowadays, discriminating between α_1- and α_2-adrenoceptors; selective α_2-adrenoceptor agonists are clonidine and UK-14,304, while yohimbine and rauwolscine are selective α_2-adrenoceptor antagonists. When noradrenaline inhibits the release of acetylcholine, it does so by acting on presynaptic inhibitory α_2-adrenoceptors on the cholinergic nerve endings. Activation of these adrenoceptors stimulates G_i-proteins. This G-protein inhibits adenylyl cyclase, resulting in a decrease in cyclic AMP synthesis and a decreased calcium influx *via* blockade of the N-type calcium channels; the α_2-adrenoceptors can also directly open potassium channels. These two mechanisms result in a reduced transmitter release *via* hyperpolarisation.

Many studies, both contractility and release studies, demonstrated the presence of inhibitory α_2-adrenoceptors on the cholinergic neurones innervating the guinea-pig ileum (e.g. Funk *et al.*, 1995; Colucci *et al.*, 1998) and distal colon (Marcoli *et al.*, 1989; Giaroni *et al.*, 1999). Contractility experiments suggested the presence of presynaptic inhibitory α_2-adrenoceptors on the cholinergic neurones innervating the circular muscle from the human distal oesophageal body (Tøttrup *et al.*, 1990); in human taenia coli, there is some evidence that presynaptic α-adrenoceptors are present inhibiting the release of acetylcholine (Del Tacca *et al.*, 1970), however, it is not yet known with certainty whether these are α_2-adrenoceptors. At the level of the stomach, it has also been demonstrated that noradrenaline reduces the release of acetylcholine. *In vivo* experiments showed that stimulation of noradrenergic

neurones inhibited contractions in the cat stomach induced by stimulation of vagal cholinergic neurones (Jansson and Martinson, 1966; Jansson and Lisander, 1969). *In vitro*, α_2-adrenoceptors have been shown to be present on postganglionic cholinergic neurones in the canine and rat gastric fundus (Lefebvre *et al.*, 1984; Verplanken *et al.*, 1984; MacDonald *et al.*, 1990); there is also evidence that presynaptic α_2-adrenoceptors are present on the intrinsic cholinergic neurones of guinea-pig gastric corpus and antrum (Schemann, 1991; Tack and Wood, 1992).

1.3.3. Presynaptic modulation via 5-HT$_4$-receptors

In the GI tract, 5-hydroxytryptamine (5-HT) is present in high concentrations in the enterochromaffin cells (ECs) of the epithelium, although some serotonergic neurones are also present (Racké *et al.*, 1991, 1996). The luminal content is separated from the afferent nerve endings by the mucosal epithelium. Luminal chemicals or increased intraluminal pressure cause the release of 5-HT from ECs. The released 5-HT might act as an essential intermediate in reflexes initiated from the mucosa whereby 5-HT is thought to stimulate the IPANs to trigger the peristaltic reflex *via* stimulation of 5-HT-receptors. Seven different 5-HT-receptor classes have been identified, and some of these can be further divided into subclasses, including 5-HT$_{1A}$-, 5-HT$_{1B}$-, 5-HT$_{1D}$-, 5-ht$_{1E}$-, 5-ht$_{1F}$-, 5-HT$_{2A}$-, 5-HT$_{2B}$-, 5-HT$_{2C}$-, 5-HT$_3$-, 5-HT$_4$-, 5-ht$_{5A}$-, 5-ht$_{5B}$-, 5-HT$_6$- and 5-HT$_7$-receptors (Hoyer *et al.*, 1994; Gerhardt & van Heerikhuizen, 1997). When the genes for certain 5-HT-receptors are cloned, but there function is not yet known, then these receptors are indicated as 5-ht-receptors. Stimulation of some of these receptors influences GI motility. For instance, 5-HT$_3$-receptor antagonists were shown to moderately accelerate gastric emptying in man (Akkermans *et al.*, 1988), delay colonic transit in humans (Houghton *et al.*, 2000) and lengthen the MMC time interval in rat small intestine (Lördal & Hellstrom, 1999) suggesting that 5-HT$_3$-receptors play a stimulatory or inhibitory role in the control of GI motility, depending on the region of the GI tract.

An important 5-HT-receptor in the GI tract is the 5-HT$_4$-receptor, described for the first time in mouse embryo colliculi neurones (Dumuis *et al.*, 1988). 5-HT$_4$-receptors are positively linked with adenylyl cyclase, increasing the concentration of cyclic AMP (Hoyer *et al.*, 1994; Gerhardt & van Heerikhuizen, 1997). Both selective 5-HT$_4$-receptor agonists, e.g. prucalopride (Briejer *et al.*, 2001), and antagonists, e.g. SB204070 (Wardle *et al.*, 1994) and GR113808 (Gale *et al.*, 1994), are available. These two antagonists are very potent and selective (Ford & Clarke, 1993), especially GR113808 that lacks antagonist affinity at other

5-HT-receptor subtypes, except at the 5-HT_3-receptor although the affinity is much lower than at the 5-HT_4-receptor (Gale *et al.*, 1994). In the GI tract, 5-HT_4-receptors can have a clearly distinct location and function. Muscular 5-HT_4-receptors are responsible for the relaxation of smooth muscle cells. Relaxant 5-HT_4-receptors are demonstrated on the circular muscle cells of canine rectum (Prins *et al.*, 1999), human colon (Tam *et al.*, 1994, 1995; McLean & Coupar, 1996; Prins *et al.*, 2000b) and rat oesophagus and ileum (Baxter *et al.*, 1991; Tuladhar *et al.*, 1996). Other 5-HT_4-receptors are located on cholinergic neurones, increasing the release of acetylcholine. In the stomach, facilitatory 5-HT_4-receptors on cholinergic neurones have been demonstrated in guinea-pig and rat (Buchheit & Bult, 1994; Amemiya *et al.*, 1996; Matsuyama *et al.*, 1996), whereby a recent study suggests a regional distribution of neuronal 5-HT_4-receptors in the guinea-pig stomach, 5-HT_4-receptors being present in the corpus and antrum but absent in the fundus (Takada *et al.*, 1999). It might be expected that an increased release of acetylcholine due to stimulation of 5-HT_4-receptors increases gastric emptying; indeed, *in vivo* cisapride and prucalopride, two 5-HT_4-receptor agonists, accelerated human stomach emptying (Johnson, 1989; Bouras *et al.*, 2001), although in an earlier study, Bouras and colleagues (1999) did not find any evidence that stimulation of 5-HT_4-receptors accelerates human gastric emptying.

Facilitatory 5-HT_4-receptors have also been demonstrated on cholinergic neurones innervating the longitudinal muscle layer of guinea-pig, human and canine colon (Elswood *et al.*, 1991; Prins *et al.*, 2000a), but not on cholinergic neurones innervating the circular muscle layer, where 5-HT_4-receptors are located on the smooth muscle cells, causing relaxation (Tam *et al.*, 1994, 1995; McLean & Coupar, 1996; Prins *et al.*, 2000b). *In vivo*, prucalopride stimulates colonic transit both in healthy people and patients with constipation (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999, 2001). This is difficult to explain as the relaxant 5-HT_4-receptors on the circular muscle would predict a decrease in colonic transit, and the increased contractility of the longitudinal muscle due to the stimulation of the 5-HT_4-receptors on the cholinergic neurones is not expected to induce sufficient force to increase transit. The precise mechanism of stimulation of colonic transit by 5-HT_4-receptors is thus not known yet.

1.3.4. Interaction between the cholinergic and nitrergic innervation

Many GI smooth muscle cells are innervated by both cholinergic and nitrergic neurones. The cholinergic neurones release the neurotransmitter acetylcholine, that contracts the muscle cells by activating postsynaptic muscarinic receptors. Nitrergic neurones release

the relaxant NO. It can thus be expected that NO and acetylcholine will counteract each other at the postsynaptic level by functional antagonism. It is therefore difficult to determine in functional studies measuring smooth muscle activity whether NO also inhibits presynaptically the release of acetylcholine from the cholinergic nerve endings. Therefore, it is important to measure the release of acetylcholine directly. The spontaneous and stimulated release of acetylcholine from cholinergic neurones of tissues can be measured in the presence of a acetylcholinesterase inhibitor, preventing the breakdown of acetylcholine. Since the amount of acetylcholine released in the organ bath is too low to measure as such, it is necessary to concentrate the samples by lyophilization, before it is possible to analyse them by chromatography (e.g. Soejima *et al.*, 1993; Shen & Mitchelson, 2001). Therefore, most often tissues are incubated with [^{3}H]-choline to allow the cholinergic neurones to synthesise [^{3}H]-acetylcholine. Without concentrating the samples, the released radioactivity upon neuronal activation can easily be measured and the different radioactive components released can be separated by HPLC to determine to what extent [^{3}H]-acetylcholine accounts for the radioactivity released.

In guinea-pig, mouse and canine small intestine, NOS inhibitors were shown to increase the electrically-evoked [^{3}H]-acetylcholine release without interfering with the basal release (Hryhorenko *et al.*, 1994; Kilbinger & Wolf, 1994; Kilbinger, 1996; Mang *et al.*, 2000). NO donors, however, were shown to increase basal release but to inhibit the electrically-evoked release of [^{3}H]-acetylcholine in guinea-pig ileum (Wiklund *et al.*, 1993; Hebeiβ & Kilbinger, 1996) and to inhibit electrically-evoked [^{3}H]-acetylcholine release in mouse ileum (Mang *et al.*, 2000). The effect of NO on basal and electrically-induced release of acetylcholine is probably mediated *via* activation of soluble guanylyl cyclase. Indeed, ODQ, a selective inhibitor of NO-sensitive soluble guanylyl cyclase (Garthwaite *et al.*, 1995), prevented the stimulatory effect of NO donors on basal [^{3}H]-acetylcholine release in guinea-pig ileum (Hebeiβ & Kilbinger, 1996), suggesting that NO stimulates basal acetylcholine release from myenteric neurones through activation of guanylyl cyclase. ODQ facilitated electrically-induced release of [^{3}H]-acetylcholine in guinea-pig ileum, an effect that was prevented by NOS inhibitors (Hebeiss & Kilbinger, 1998), suggesting that endogenous NO activates soluble guanylyl cyclase, leading to inhibition of acetylcholine release. YC-1, a NO-independent activator of soluble guanylyl cyclase (Wu *et al.*, 1995), indeed inhibited the electrically-induced [^{3}H]-acetylcholine release in guinea-pig ileum (Hebeiss & Kilbinger, 1998). Also in mouse ileum, ODQ stimulated electrically-evoked [^{3}H]-acetylcholine release

(Mang *et al.*, 2000). These results indicate that NO interferes with acetylcholine release *via* soluble guanylyl cyclase.

In other GI tissues however, no evidence was found that NO interferes with acetylcholine release. In guinea-pig taenia coli, the release of [^{3}H]-acetylcholine was unaffected by either a NOS inhibitor or a NO donor (Ward *et al.*, 1996), arguing against a presynaptic effect of NO on acetylcholine release. Also in canine proximal colon and opossum lower oesophageal sphincter, no evidence was found in favour of a presynaptic action of NO on cholinergic nerve endings (Cellek & Moncada, 1997; Rae *et al.*, 1998), indicating that NO might only interfere with cholinergic neurotransmission by functional antagonism at the postsynaptic level.

At the level of the stomach, functional experiments suggested that NO inhibits acetylcholine release from cholinergic neurones. NOS inhibitors were shown to enhance electrically-induced cholinergic contractions in smooth muscle strips of the rat and rabbit gastric fundus (Lefebvre *et al.*, 1992b; Baccari *et al.*, 1993, 1994) and contractions of the rabbit stomach, induced by vagal stimulation *in vivo* (Iversen *et al.*, 1997). An *in vivo* experiment in dog gastric fundus suggested that NO inhibits presynaptically acetylcholine release from cholinergic nerve endings as the NOS inhibitor L-NNA increased fundic tone, an effect that was prevented by prior atropine administration (Paterson *et al.*, 2000). However, these functional experiments cannot exclude that NO solely interferes with cholinergic contractions by functional antagonism of acetylcholine at the level of the muscle cells. In guinea-pig gastric fundus, conflicting results were found as Sotirov and colleagues (1999) described that L-NNA inhibited electrically-induced [^{3}H]-acetylcholine release, indicating that NO facilitates acetylcholine release. Milenov & Kalfin (1996), however, could not find evidence for a nitrergic modulation of acetylcholine release in guinea-pig gastric fundus as L-NAME was without effect on [^{3}H]-acetylcholine release.

As mentioned before, VIP is often co-released with NO, and activation of presynaptic VIP-receptors on cholinergic neurones might inhibit the release of acetylcholine from the cholinergic nerve endings. A functional study showed that VIP inhibits cholinergic contractions in rabbit stomach (Baccari *et al.*, 1994) but this effect of VIP on the cholinergic contractions can be due to functional antagonism. In guinea-pig stomach, VIP inhibited the electrically-evoked cholinergic contractions as well as the release of [^{3}H]-acetylcholine (Milenov *et al.*, 1991); in LMMP preparations of guinea-pig ileum, VIP and pituitary adenylate cyclase-activating polypeptide (PACAP), the latter belonging to the VIP family,

increased basal and inhibited electrically-induced [^{3}H]-acetylcholine release (Katsoulis *et al.*, 1993).

1.4. References

ABRAHAMSSON, H. (1973). Studies on the inhibitory nervous control of gastric motility. *Acta Physiol. Scand.*, (Suppl. 390), 5-38

ABRAHAMSSON, H. (1986). Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch. Int. Pharmacodyn.*, **280** (Suppl.), 50-61

ADEM, A. & KARLSSON, E. (1997). Muscarinic receptor subtype selective toxins. *Life Sciences*, **60**, 1069-1076

AKKERMANS, L.M.A., VOS, A., HOEKSTRA, A., ROELOFS, J.M. & HOROWITZ, M. (1988). Effects of ICS 205-930 (a specific 5-HT_3 receptor antagonist) on gastric emptying of a solid meal in normal subjects. *Gut*, **29**, 1249-1252

AMEMIYA, N., HATTA, S., TAKEMURA, H. & OHSHIKA, H. (1996). Characterization of the contractile response induced by 5-methoxytryptamine in rat stomach fundus strips. *Eur. J. Pharmacol.*, **318**, 403-409

ANDREWS, P.L.R. (1990). Central organization of the vagal drive to the non-adrenergic non-cholinergic neurones controlling gastric motility. *Arch. Int. Pharmacodyn.*, **303**, 167-198

BACCARI, M.C., BERTINI, M. & CALAMAI, F. (1993). Effects of L-N^G-nitro arginine on cholinergic transmission in the gastric muscle of the rabbit. *Neuroreport*, **4**, 1102-1104

BACCARI, M.C., CALAMAI, F. & STADERINI, G. (1994). Modulation of cholinergic transmission by nitric oxide, VIP and ATP in the gastric muscle. *Neuroreport*, **5**, 905-908

BARBIER, A.J. & LEFEBVRE, R.A. (1993). Involvement of the L-arginine: nitric oxide pathway in non-adrenergic non-cholinergic relaxation of the cat gastric fundus. *J. Pharmacol. Exp. Ther.*, **266**, 172-178

BAUMGARTEN, H.G. (1982). Morphological basis of gastrointestinal motility: structure and innervation of gastrointestinal tract. In *Handbook of Experimental Pharmacology, vol. 59/I, Mediators and Drugs in Gastrointestinal Motility I.* ed. Bertaccini, G., Springer-Verlag Berlin, pp. 7-53

BAXTER, G.S., CRAIG, D.A. & CLARKE, D.E. (1991). 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 439-446

BENNETT, M.R. (1997). Non-adrenergic non-cholinergic (NANC) transmission to smooth muscle: 35 years on. *Progress in Neurobiol.*, **52**, 159-195

BOECKXSTAENS, G.E., PELCKMANS, P.A., DE MAN, J.G., BULT, H., HERMAN, A.G. & VAN MAERCKE, Y.M. (1992). Evidence for a differential release of nitric oxide and vasoactive intestinal polypeptide by non-adrenergic non-cholinergic nerves in the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **318**, 107-115

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368**, 850-853

BOURAS, E.P., CAMILLERI, M., BURTON, D.D. & MCKINZIE, S. (1999). Selective stimulation of colonic transit by the benzofuran 5HT$_4$ agonist, prucalopride, in healthy humans. *Gut*, **44**, 682-686

BOURAS, E.P., CAMILLERI, M., BURTON, D.D., THOMFORDE, G., MCKINZIE, S. & ZINSMEISTER, A.R. (2001). Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. *Gastroenterology*, **120**, 354-360

BRIEJER, M.R., BOSMANS, J.-P., VAN DAELE, P., JURZAK, M., HEYLEN, L., LEYSEN, J.E., PRINS, N.H. & SCHUURKES, J.A.J. (2001). The in vitro pharmacological profile of prucalopride, a novel enterokinetic compound. *Eur. J. Pharmacol.*, **423**, 71-83

BUCHHEIT, K. & BULT, T. (1994). Stimulant effects of 5-hydroxytryptamine on guinea-pig stomach preparations *in vitro*. *Eur. J. Pharmacol.*, **262**, 91-97

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469-476

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24**, 509-581

CAMILLERI, M. & FORD, M.J. (1998). Review article: colonic sensorimotor physiology in health, and its alteration in constipation and diarrhoeal disorders. *Aliment. Pharmacol. Ther.*, **12**, 287-302

CAULFIELD, M.P. & BIRDSALL, N.J.M. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.*, **50**, 279-290

CELLEK, S. & MONCADA, S. (1997). Nitrergic modulation of cholinergic responses in the opossum lower oesophageal sphincter. *Br. J. Pharmacol.*, **122**, 1043-4046

COLUCCI, R., BLANDIZZI, C., CARIGNANI, D., PLACANICA, G., LAZZERI, G. & DEL TACCA, M. (1998). Effects of imidazoline derivatives on cholinergic motility in guinea-pig ileum: involvement of presynaptic α_2-adrenoceptors or imidazoline receptors? *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **357**, 682-691

COSTA, M. & BROOKES, S.J.H. (1994). The enteric nervous system. *Am. J. Gastroenterol.*, **89**, S129-S137

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.*, **69**, 59-78

DEL TACCA, M., SOLDANI, G., SELLI, M. & CREMA, A. (1970). Action of catecholamines on release of acetylcholine from human taenia coli. *Eur. J. Pharmacol.*, **9**, 80-84

DESAI, K.M., WARNER, T.D., BISHOP, A.E., POLAK, J.M. & VANE, J.R. (1994). Nitric oxide, and not vasoactive intestinal polypeptide, as the main neurotransmitter of vagally induced relaxation of the guinea-pig stomach. *Br. J. Pharmacol.*, **113**, 1197-1202

DIETRICH, C. & KILBINGER, H. (1995). Prejunctional M1 and postjunctional M3 muscarinic receptors in the circular muscle of the guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **351**, 237-243

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.R. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **256**, 727-733

DUMUIS, A., BOUHELAL, R., SEBBEN, M., CORY, R. & BOCKAERT, J. (1988). A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol. Pharmacol.*, **34**, 880-887

EGLEN, R.M., HEDGE, S.S. & WATSON, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.*, **48**, 531-565

EHLERT, F.J., SAWYER, G.W. & ESQUEDA, E.E. (1999). Contractile role of M_2 and M_3 muscarinic receptors in gastrointestinal smooth muscle. *Life Sciences*, **64**, 387-394

ELSWOOD, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Identification of putative 5-HT_4-receptors in guinea-pig ascending colon. *Eur. J. Pharmacol.*, **196**, 149-155

EMMANUEL, A.V., KAMM, M.A., ROY, A.J. & ANTONELLI, K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut*, **42**, 511-516

FAHRENKRUG, J. (1982). VIP as a neurotransmitter in the peripheral nervous system. In *Vasoactive Intestinal Polypeptide*, ed. Said, S.I., Raven Press, New York, pp. 361-372

FORD, A.P.D.W. & CLARKE, D.E. (1993). The 5-HT_4 receptor. *Med. Res. Rev.*, **13**, 633-662

FUNK, L., TRENDELENBURG, A.-U., LIMBERGER, N. & STARKE, K. (1995). Subclassification of presynaptic α_2-adrenoceptors: α_{2D}-autoreceptors and α_{2D}-adrenoceptors modulating release of acetylcholine in guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352**, 58-66

FURNESS, J.B., BORNSTEIN, J.C., KUNZE, W.A.A. & CLERC, N. (1996). The enteric nervous system and its extrinsic connections. In *Textbook of Gastroenterology*, vol. 1, 3th edn., ed. Yamada, T., Lippincot Williams and Wilkins, Philadelphia, 11-35

FURNESS, J.B., BORNSTEIN, J.C., MURPHY, R. & POMPOLO, S. (1992). Roles of peptides in transmission in the enteric nervous system. *Trends Neurosci.*, **15**, 361-372

FURNESS, J.B. & COSTA, M. (1987). Arrangement of enteric plexuses. In *The Enteric Nervous System*., eds. Furness, J.B. & Costa, M., Churchill Livingstone, Edingburgh, 6-25

GALE, J.D., GROSSMAN, J.-P., WHITEHEAD, J.W., OXFORD, A.W., BUNCE, K.T. & HUMPHREY, P.P. (1994). GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. *Br. J. Pharmacol.*, **111**, 332-338

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.*, **48**, 184-188

GERHARDT, C.C. & VAN HEERIKHUIZEN, H. (1997). Functional characteristics of heterologously expressed 5-HT receptors. *Eur. J. Pharmacol.*, **334**, 1-23

GIARONI, C., SOMAINI, L., MARINO, F., COSENTINO, M., SENALDI, A., DE PONTI, F., LECCHINI, S. & FRIGO, G. (1999). Modulation of enteric cholinergic neurons by hetero- and autoreceptors: cooperation among inhibitory inputs. *Life Sciences*, **65**, 813-821

GOYAL, R.K. & HIRANO, I. (1996). The enteric nervous system. *New Engl. J. Med.*, **334**, 1106-1115

GRIMM, U., MOSER, E., MUTSCHLER, M.E. & LAMBRECHT, G. (1994). Muscarinic receptors: focus on presynaptic mechanisms and recently developed novel agonists and antagonists. *Pharmazie*, **49**, 711-726

GRUNDY, D., GHARIB-NASERI, M.K. & HUTSON, D. (1993). Role of nitric oxide and vasoactive intestinal polypeptide in vagally mediated relaxation of the gastric corpus in the anaesthetized ferret. *J. Autonom. Nerv. Syst.*, **43**, 241-246

HASLER, W.L. (1996a). The physiology of gastric motility and gastric emptying. In *Textbook of Gastroenterology*, vol. 1, 3th edn., ed. Yamada, T., Lippincot Williams and Wilkins, Philadelphia, pp. 188-215

HASLER, W.L. (1996b). Motility of the small intestine and colon. In *Textbook of Gastroenterology*, vol. 1, 3th edn., ed. Yamada, T., Lippincot Williams and Wilkins, Philadelphia, pp. 215-245

HEBEIβ, K. & KILBINGER, H. (1996). Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. *Br. J. Pharmacol.*, **118**, 2073-2078

HEBEISS, K. & KILBINGER, H. (1998). Nitric oxide-sensitive guanylyl cyclase inhibits acetylcholine release and excitatory motor transmission in the guinea-pig ileum. *Neuroscience*, **82**, 623-629

HILLEMEIER, C. (1995). An overview of the effects of dietary fiber on gastrointestinal transit. *Pediatrics*, **96**, 997-999

HOUGHTON, L.A., FOSTER, J.M. & WHORWELL, P.J. (2000). Alosetron, a 5-HT_3 receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers. *Aliment. Pharmacol. Ther.*, **14**, 775-782

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46**, 157-203

HRYHORENKO, L.M., WOSKOWSKA, Z. & FOX-THRELKELD, J.-A.E.T. (1994). Nitric oxide (NO) inhibits release of acetylcholine from nerves of isolated circular muscle of the canine ileum: relationship to motility and release of nitric oxide. *J. Pharmacol. Exp. Ther.*, **271**, 918-926

IVERSEN, H.H., CELSING, F., LEONE, A.M., GUSTAFSSON, L.E. & WIKLUND, N.P. (1997). Nerve-induced release of nitric oxide in the rabbit gastrointestinal tract as measured by in vivo microdialysis. *Br. J. Pharmacol.*, **120**, 702-706

JANSSON, G., LISANDER, B. (1969). On adrenergic influence on gastric motility in chronically vagotomized cats. *Acta Phys. Scand.*, **76**, 463-471

JANSSON, G., MARTINSON, J. (1966). Studies on the ganglionic site of action of sympathetic outflow to the stomach. *Acta Phys. Scand.*, **68**, 184-192

JOHNSON, A.G. (1989). The effects of cisapride on antroduodenal co-ordination and gastric emptying. *Scand. J. Gastroenterol.*, **24**, 36-43

JUNCQUEIRA, L.C., CARNEIRO, J. & KELLY, R.O. (Bewerkt door James, J., Nieuwenhuis, P. & Wisse, E.). (1993). Het spijsverteringskanaal. In *Functionele histology*, 6de edn., pp. 355-389

KATSOULIS, S., CLEMENS, A., SCHWÖRER, H., CREUTZFELDT, W. AND SCHMIDT, W.E. (1993). PACAP is a stimulator of neurogenic contraction in guinea pig ileum. *Am. J. Physiol.*, **265**, G295-G302

KELLY, K.A. (1980). Gastric emptying of liquids and solids: roles of proximal and distal stomach. *Am. J. Physiol.*, **239**, G71-G76

KILBINGER, H. (1996). Modulation of acetylcholine release by nitric oxide. *Progr. Brain Res.*, **109**, 219-224

KILBINGER, H. & WOLF, D. (1994). Increase by NO synthase inhibitors of acetylcholine release from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349**, 543-545

KOSTKA, P., KWAN, C.-Y. & DANIEL, E.E. (1989). Presynaptic and postsynaptic muscarinic receptors in dog ileum: binding studies. *Eur. J. Pharmacol.*, **173**, 35-42

KUNZE, W.A.A. & FURNESS, J.B. (1999). The enteric nervous system and regulation of intestinal motility. *Annu. Rev. Physiol.*, **61**, 117-142

LEFEBVRE, R.A. (1993). Non-adrenergic non-cholinergic neurotransmission in the proximal stomach. *Gen. Pharmacol.*, **24**, 270-278

LEFEBVRE, R.A., BAERT, E. & BARBIER, A.J. (1992a). Influence of L-N^G-nitroarginine on non-adrenergic non-cholinergic relaxation in the guinea-pig gastric fundus. *Br. J. Pharmacol.*, **106**, 173-179

LEFEBVRE, R.A., DE VRIESE, A. & SMITS, G.J.M. (1992b). Influence of vasoactive intestinal polypeptide and N^G-nitro-L-arginine methyl ester on cholinergic neurotransmission in the rat gastric fundus. *Eur. J. Pharmacol.*, **221**, 235-242

LEFEBVRE, R.A., SMITS, G.J.M. & TIMMERMANS, J.-P. (1995). Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. *Br. J. Pharmacol.*, **116**, 2017-2026

LEFEBVRE, R.A., WILLEMS, J.L. & BOGAERT, M.G. (1984). Inhibitory effect of dopamine on canine gastric fundus. *Naunyn Schmied. Arch. Pharmacol.*, **326**, 22-28

LI, G.C. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303-309

LÖRDAL, M. & HELLSTROM, P.M. (1999). Serotonin stimulates migrating myoelectric complex *via* 5-HT_3-receptors dependent on cholinergic pathways in rat small intestine. *Neurogastroenterol. Mot.*, **11**, 1-10

MACDONALD, A., KELLY, J. & DETTMAR, P.W. (1990). Pre-and postsynaptic α-adrenoceptor-mediated responses in the rat gastric fundus in-vitro. *J. Pharm Pharmacol.*, **42**, 752-757

MACLEAN, P.G. & COUPAR, I.M. (1996). Further investigation into the signal transduction mechanism of the 5-HT_4-like receptor in the circular smooth muscle of human colon. *Br. J. Pharmacol.*, **118**, 1058-1064

MANG, C.F., TRÜMPLER, S. & KILBINGER, H. (2000). Inhibition by endogenous nitric oxide of acetylcholine release in the mouse isolated ileum. *Br. J. Pharmacol.*, **131**, 32P

MARCOLI, M., DE PONTI, F., LECCHINI, S., CREMA, A. & FRIGO, G.M. (1989). [^{3}H]acetylcholine release from the guinea-pig distal colon: comparison with ileal [^{3}H]acetylcholine release and effect of adrenoceptor stimulation. *J. Pharm. Pharmacol.*, **41**, 824-828

MATSUYAMA, S., SAKIYAMA, H., NEI, K. & TANAKA, C. (1996). Identification of putative 5-hydroxytryptamine$_4$ (5-HT_4) receptors in guinea pig stomach: the effect of TKS159, a novel agonist, on gastric motility and acetylcholine release. *J. Pharmacol. Exp. Ther.*, **276**, 989-995

MILENOV, K. & KALFIN, R. (1996). Cholinergic-nitrergic interactions in the guinea-pig gastric fundus. *Neuropeptides*, **30**, 365-371

MILENOV, K., KALFIN, R. & MANDREK, K. (1991). Effect of vasoactive intestinal peptide (VIP) on the mechanical activity and [^{3}H] acetylcholine release in guinea-pig gastric muscle. *Acta Physiol. Pharmacol. Bulg.*, **17**, 13-18

OGISHIMA, M., KAIBARA, M., UEKI, S., KURIMOTO, T. & TANIYAMA, K. (2000). Z-338 facilitates acetylcholine release from enteric neurons due to blockade of muscarinic autoreceptors in guinea pig stomach. *J. Pharmacol. Exp. Ther.*, **294**, 33-37

PATERSON, C.A., ANVARI, M., TOUGAS, G. & HUIZINGA, J.D. (2000). Nitrergic and cholinergic vagal pathways involved in the regulation of canine proximal gastric tone: an *in vivo* study. *Neurogastroenterol. Mot.*, **12**, 301-306

POLLOCK, J.S., FÖRSTERMANN, U., MITCHELL, J.A., WARNER, T.D., SCHMIDT, H.H.H.M., NAKANE, M. & MURAD, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA*, **88**, 10480-10484

PRINS, N.H., VAN HASELEN, J.F.W.R., LEFEBVRE, R.A., BRIEJER, M.R., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (1999). Pharmacological characterisation of canine isolated rectum circular smooth muscle. *Br. J. Pharmacol.*, **127**, 1431-1437

PRINS, N.H., AKKERMANS, L.M.A., LEFEBVRE, R.A. & SCHUURKES, J.A.J. (2000a). 5-HT_4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br. J. Pharmacol.*, **131**, 927-932

PRINS, N.H., SHANKLEY, N.P., WELSH, N.J., BRIEJER, M.R., LEFEBVRE, R.A., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (2000b). An improved *in vitro* bioassay for the study of 5-HT_4 receptors in the human isolated large intestinal circular muscle. *Br. J. Pharmacol.*, **129**, 1601-1608

RACKÉ, K., REIMANN, A., SCHWÖRER, H. & KILBINGER, H. (1996). Regulation of 5-HT release from enterochromaffin cells. *Beh. Brain Res.*, **73**, 83-87

RACKÉ, K. & SCHWÖRER, H. (1991). Regulation of serotonin release from the intestinal mucosa. *Pharmacol. Res.*, **23**, 13-25

RAE, M.G., KHOYI, M.A. & KEEF, K.D. (1998). Modulation of cholinergic neuromuscular transmission by nitric oxide in canine colonic circular smooth muscle. *Am. J. Physiol.*, **275**, G1324-G1332

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262**, G379-G392

SCHEMANN, M. (1991). Excitatory and inhibitory effects of norepinephrine on myenteric neurons of the guinea-pig gastric corpus. *Pflügers Arch.*, **418**, 575-580

SCHMIDT, H.H.H.W., LOHMANN, S.M. & WALTER, U. (1993). Minireview. The nitric oxide and cGMP signal transduction system: regulation and mecanism of action. *Bioch. Biophys. Acta*, **1178**, 153-175

SHEN, A. & MITCHELSON, F. (2001). Characterization of the prejunctional inhibitory muscarinic receptor on cholinergic nerves in the rat urinary bladder. *Eur. J. Pharmacol.*, **2001**, 179-187

SHI, X.-Z. & SARNA, S.K. (1997). Inflammatory modulation of muscarinic receptor activation in canine ileal circular muscle cells. *Gastroenterology*, **112**, 864-874

SHI, X.-Z. & SARNA, S.K. (1999). Differential inflammatory modulation of canine ileal longitudinal and circular muscle cells. *Am. J. Physiol.*, **277**, G341-G350

SHUTTLEWORTH, C.W.R. & KEEF, K.D. (1995). Roles of peptides in enteric neuromuscular transmission. *Reg. Peptides*, 56, 101-120

SOEJIMA, O., KATSURAGI, T. & FURUKAWA, T. (1993). Opposite modulation by muscarinic M_1 and M_3 receptors of acetylcholine release from guinea pig ileum as measured directly. *Eur. J. Pharmacol.*, **249**, 1-6

SOTIROV, E., PAPASOVA, M. & SÁNTHA, E. (1999). Nitric oxide (NO) increases acetylcholine release from and inhibits smooth muscle contraction of guinea-pig gastric fundus. *Brain Res. Bull.*, **49**, 297-302

TACK, J.F. & WOOD, J.D. (1992). Actions of noradrenaline on myenteric neurones in the guinea-pig antrum. *J. Auton. Nerv. Syst.*, **41**, 67-78

TAKADA, K., SAKURAI-YAMASHITA, Y., YAMASHITA, K., KAIBARA, M., HAMADA, Y., NAKANE, Y., HIOKI, K. & TANIYAMA, K. (1999). Regional difference in correlation of 5-HT_4-receptor distribution with cholinergic transmission in the guinea-pig stomach. *Eur. J. Pharmacol.*, **374**, 489-494

TAM, F.S.-F., HILLIER, K. & BUNCE, K.T. (1994). Characterization of the 5-hydroxytryptamine receptor type involved in inhibition of spontaneous activity of human isolated colonic circular muscle. *Br. J. Pharmacol.*, **113**, 143-150

TAM, F.S.-F., HILLIER, K., BUNCE, K.T. & GROSSMAN, C. (1995). Differences in response to 5-HT_4 receptor agonists and antagonists of the 5-HT_4-like receptor in human colon circular smooth muscle. *Br. J. Pharmacol.*, **115**, 172-176

TODA, N., MONCADA, S., FURCHGOTT, R. & HIGGS, E.A. (2000). In *Nitric oxide and the peripheral nervous system*., Portland Press Ltd, Pp. 1-200

TONINI, M., DE GIORGIO, R., DE PONTI, F., STERNINI, C., SPELTA, V., DIONIGI, P., BARBARA, G., STANGHELLINI, V. & CORINALDESI, R. (2000). Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations. *Br. J. Pharmacol.*, **129**, 12-20

TØTTRUP, A., FORMAN, A., FUNHC-JENSEN, P., RAUNDHAL, U. & ANDERSSON, K.E. (1990). Effects of transmural field stimulation in isolated muscle strips from human esophagus. *Am. J. Physiol.*, **258**, G344-G351

TULADHAR, B.R., COSTALL, B. & NAYLOR, R.J. (1996). Pharmacological characterization of the 5-hydroxytryptamine receptor mediating relaxation in the rat isolated ileum. *Br. J. Pharmacol.*, **119**, 303-310

VERPLANKEN, P.A., LEFEBVRE, R.A. & BOGAERT, M.G. (1984). Pharmacological characterization of *alpha* adrenoceptors in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **231**, 404-410

WARD, S.M., DALZIEL, H.H., KHOYI, M.A., WESTFALL, A.S., SANDERS, K.M. & WESTFALL, D.P. (1996). Hyperpolarization and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli. *Br. J. Pharmacol.*, **118**, 49-56

WARDLE, K.A., ELLIS, E.S., BAXTER, G.S., KENNETT, G.A., GASTER, L.M. & SANGER, G.J. (1994). The effects of SB 204070, a highly potent and selective 5-HT_4 receptor antagonist, on guinea-pig distal colon. *Br. J. Pharmacol.*, **112**, 789-794

WIKLUND, C.U., OLGART, C., WIKLUND, N.P. & GUSTAFSSON, L.E. (1993). Modulation of cholinergic and substance P-like neurotransmission by nitric oxide in the guinea-pig ileum. *Br. J. Pharmacol.*, **110**, 833-839

WU, C.C., KO, F.N., KUO, S.C., LEE, F.Y. & TENG, C.M. (1995). YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.*, **116**, 1973-1978

CHAPTER 2

INVESTIGATION OF THE INTERACTION BETWEEN CHOLINERGIC AND NITRERGIC NEUROTRANSMISSION IN THE PIG GASTRIC FUNDUS

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CHAPTER 2

INVESTIGATION OF THE INTERACTION BETWEEN CHOLINERGIC AND NITRERGIC NEUROTRANSMISSION IN THE PIG GASTRIC FUNDUS

2.1 Summary

The interaction between the cholinergic and nitrergic innervation was investigated in circular muscle strips of the pig gastric fundus. In physiological salt solution containing $4x10^{-6}$ M guanethidine, electrical field stimulation (EFS; 40 V, 0.5 ms, 0.5 - 32 Hz, 10 s at 4 min intervals) induced small transient relaxations at 0.5 - 4 Hz, and large frequency-dependent contractions, sometimes followed by off-relaxations, at 8 - 32 Hz. In the presence of L-N^G-nitroarginine methyl ester (L-NAME; $3x10^{-4}$ M) or physostigmine (10^{-6} M), relaxations were reversed into contractions and contractions were enhanced. Physostigmine added to L-NAME further enhanced contractions, while addition of L-NAME to physostigmine had no additional effect. Off-relaxations were enhanced in the presence of L-NAME and physostigmine. L-NAME and physostigmine consistently increased basal tone. Tissues contracted by 5-hydroxytryptamine or by acetylcholine responded to EFS in a similar way as in basal conditions and L-NAME reversed the relaxations at the lower stimulation frequencies into contractions and enhanced the contractions at the higher stimulation frequencies. Off-relaxations in the presence of L-NAME were partially reduced by α-chymotrypsin (10 U ml^{-1}). In the absence of physostigmine, the concentration-response curve to exogenous acetylcholine was not influenced by L-NAME. Contractions of the same amplitude induced by EFS at 4 Hz and by exogenous acetylcholine were either decreased or enhanced to the same extent by sodium nitroprusside (SNP; 10^{-5} M), depending upon the degree of relaxation by SNP. These experiments suggest that endogenous nitric oxide interferes with cholinergic neurotransmission in the pig gastric fundus by functional antagonism at the postjunctional level. The interaction is independent of the degree of contraction.

2.2. Introduction

The gastric fundus is innervated both by excitatory cholinergic neurones and by non-adrenergic non-cholinergic (NANC) inhibitory neurones, the latter being the final

effectors of the vagally mediated gastric receptive relaxation (Abrahamsson, 1986). Both vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) have been proposed as NANC neurotransmitters in the proximal part of the stomach (Lefebvre, 1993). Whereas in species such as the rat and the ferret, NO is mainly involved in short-lasting relaxations and in initiating sustained relaxations (Li & Rand, 1990; D'Amato *et al.*, 1992; Grundy *et al.*, 1993), in other species such as the guinea-pig it is also the predominant neurotransmitter during sustained relaxation (Lefebvre *et al.*, 1992a; Desai *et al.*, 1994). The vagal preganglionic efferent fibers to the stomach seem centrally organised in a reciprocal manner: when the efferents supplying the intramural cholinergic neurones are active, the discharge in those supplying the intramural inhibitory NANC neurones is suppressed, and *vice versa* (Andrews, 1990). Besides this central interaction between the two pathways, interaction might also occur between the nitrergic and cholinergic system at the level of the stomach. NO synthase inhibitors were shown to enhance electrically-induced cholinergic contractions in smooth muscle strips of the rat, guinea-pig and rabbit gastric fundus (Lefebvre *et al.*, 1992b; Baccari *et al.*, 1993; Milenov & Kalfin, 1996) and contractions of the rabbit stomach, induced by vagal stimulation *in vivo* (Iversen *et al.*, 1997). In the guinea-pig small intestine, NO donors were shown to increase the basal release but to inhibit the electrically-induced release of [^{3}H]-acetylcholine (Hebeiβ & Kilbinger, 1996).

The pig is a good non-primate model for studying human digestive function in view of the similarity of the morphology and physiology of the gastrointestinal tracts (Miller & Ullrey, 1987). The pig gastric fundus is innervated by excitatory cholinergic and inhibitory NANC neurones (Ohga & Taneike, 1977; Miyazaki *et al.*, 1991) and NO is a major contributor to NANC relaxation (Lefebvre *et al.*, 1995). The aim of this study in the pig gastric fundus was to investigate the interaction between the cholinergic and nitrergic innervation.

2.3. Methods

2.3.1. Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the pig gastric fundus. The stomach was removed from healthy castrated male pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution

(PSS). After the mucosa was removed, strips of approximately 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular muscle, with a maximum of eight strips from one pig gastric fundus. All strips were used the same day. Strips were mounted vertically between two platinum plate electrodes under a load of 2 g in 20 ml organ baths containing 20 ml of PSS (mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$), maintained at 37°C and gassed with carbogen. Guanethidine ($4x10^{-6}$ M) was present in the medium throughout all experiments. The mechanical activity of the preparations was recorded *via* isotonic transducers (T_3, Palmer Bioscience, U.S.A.) on a recorder (FWR 3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Japan). Electrical field stimulation (EFS) was applied by means of a S88 stimulator (Grass, U.S.A.). The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment.

2.3.2. Evaluation of the cholinergic-nitrergic interaction

In all these experiments, strips were first maximally contracted with 80 mM KCl, followed by rinsing every 10 min during 30 min. EFS was applied to the tissues (40 V, 0.5 ms, 10 s trains, 0.5 - 32 Hz, with an interval of 4 min). Two, or in some experiments three, frequency-response curves were obtained with an interval of at least 1 h in between. The tissues were incubated in the presence of agents for 30 min. The responses in parallel time control experiments were reproducible unless otherwise stated.

A first set of experiments was performed to study the effect of L-N^G-nitroarginine methyl ester (L-NAME), physostigmine, and their combination on the electrically-induced responses. Three frequency-response curves were obtained in two parallel tissues. Physostigmine (10^{-6} M) or L-NAME ($3x10^{-4}$ M) was added before the second curve. Before the third curve, L-NAME was added to the medium containing physostigmine, and physostigmine to the medium containing L-NAME. To study the influence of tetrodotoxin (TTX; $3x10^{-6}$ M), atropine (10^{-6} M) and hexamethonium ($5x10^{-4}$ M), two frequency-response curves were obtained before and after addition of these agents. This was done in three conditions: PSS only containing guanethidine as in all experiments, and PSS where in addition to guanethidine L-NAME ($3x10^{-4}$ M) or L-NAME ($3x10^{-4}$ M) plus physostigmine (10^{-6} M) were present from the beginning of the experiment. The influence of TTX was also studied in tissues, only stimulated at 16 and 32 Hz.

As EFS induced off-relaxations at the higher frequencies of stimulation, that were even enhanced in the presence of L-NAME, the influence of 10 U ml^{-1} α-chymotrypsin was studied on these off-relaxations in the presence of L-NAME. As α-chymotrypsin induced a pronounced contraction, the off-relaxations were also evaluated in parallel tissues, contracted to the same extent by use of 5-hydroxytryptamine (5-HT).

As it has been shown that the cholinergic-nitrergic interaction can be influenced by the degree of contraction of the tissues and by the contractile agent used (Baccari *et al.*, 1997), frequency-response curves were also obtained in tissues contracted with 5-HT or acetylcholine. In a first set, after the maximal contraction with KCl and rinsing, the tissues were contracted with $3x10^{-7}$ M 5-HT and, once a stable plateau was obtained, electrically stimulated (40 V, 0.5 ms, 10 s, 0.5 - 32 Hz at 4 min interval). This cycle was repeated twice. L-NAME ($3x10^{-4}$ M) or atropine (10^{-6} M) was added before the second cycle. Before the third cycle, atropine was added to the medium already containing L-NAME and L-NAME to the medium containing atropine. In a second set, tissues were contracted with acetylcholine in a concentration to mimic the contraction amplitude obtained with $3x10^{-7}$ M 5-HT in the previous set. In these conditions, the influence of $3x10^{-4}$ M L-NAME was studied on the electrically-induced responses.

Cumulative concentration-response curves to acetylcholine (10^{-9} - $3x10^{-4}$ M) were obtained before and after (30 min) addition of $5x10^{-4}$ M hexamethonium and $3x10^{-4}$ M L-NAME. The interval between the two curves was at least 1 h 30 min. The influence of L-NAME on exogenous acetylcholine was also studied in the continuous presence of 10^{-6} M physostigmine.

To study the influence of sodium nitroprusside (SNP) on contractions induced by EFS and exogenous acetylcholine, the following protocol was used. L-NAME ($3x10^{-4}$ M) was present throughout the experiment. Tissues were stimulated three times (40 V, 0.5 ms, 4 Hz, 10 s, 4 min interval); after rinsing, acetylcholine was administered in increasing concentrations to select the concentration, inducing a similar contraction amplitude as EFS at 4 Hz. After another rinsing interval, this concentration was repeated. After further rinsing, six parallel tissues received SNP (10^{-5} M; 4 strips) or its solvent (2 strips). In three of the SNP-treated tissues, acetylcholine was administered when the maximal relaxation by SNP was reached or 20 or 40 min later respectively. The control solvent-treated tissue was contracted three times with acetylcholine, with rinsing in between, at moments corresponding to the administration of acetylcholine in the three parallel strips. In the fourth SNP-treated tissue, EFS was applied 15 times at 4 Hz (10 s) with 4 min intervals, starting when the

maximal relaxation by SNP was reached. The control solvent-treated tissue was stimulated at the same moments as the SNP-treated tissues.

2.3.3. Data analysis

Experimental data are expressed as means ± s.e.mean and *n* refers to the number of the tissues from different animals. All results are expressed as percentage of the maximal KCl-induced contraction. When the response during electrical stimulation was not monophasic, the amplitude of the first phase is taken into account. Off-relaxations are measured from the tone level present before the start of the stimulation until maximal relaxation. Results within the same tissues were compared by the paired *t*-test or by ANOVA followed by a *t*-test corrected for multiple comparisons (Bonferroni procedure) when more than two responses had to be compared; results in different groups of tissues were compared by the unpaired *t*-test. *P* values of less than 0.05 were considered statistically significant.

2.3.4. Drugs used

Acetylcholine chloride, atropine sulphate, guanethidine sulphate, L-N^G-nitroarginine methyl ester and sodium nitroprusside were obtained from Sigma (St. Louis, U.S.A.), 5-hydroxytryptamine creatinine monosulphate from Janssen Chimica (Geel, Belgium), physostigmini salicylas from Federa (Brussels, Belgium), tetrodotoxin from Alomone labs (Jerusalem, Israel) and from Sigma.

Drugs were dissolved and diluted with distilled water. Stock solutions of 10^{-3} M TTX were kept frozen at -20°C and dilutions were made the day of the experiment.

2.4. Results

The tone of the tissues decreased during the first 30 min of the equilibration period, after which tone tended to increase during the course of the experiment. In control tissues, the responses to three consecutive frequency-response curves (40 V, 0.5 ms, 0.5 - 32 Hz, 10 s, 4 min interval) were reproducible (n = 14). EFS induced small transient relaxations at the lowest frequencies (0.5 - 4 Hz). After the 10 s period of stimulation, tone returned to the pre-stimulation level at a slower rate than it had declined. Occasionally, the relaxation was

preceded by a very small contraction. Stimulation at the highest frequencies (8 - 32 Hz) induced large fast frequency-dependent contractions (Figure 2.1A and 2.2A,B). After stimulation, tone declined as quickly as it had risen and sometimes, it decreased to a lower level than present before stimulation. These decreases of tone will be indicated as off-relaxations.

2.4.1. Influence of L-NAME and physostigmine on the electrically-induced responses

The administration of $3x10^{-4}$ M L-NAME induced an increase in resting tone of the tissues (31 ± 5 %, *n* = 14). L-NAME ($3x10^{-4}$ M) reversed the relaxations at the lowest stimulation frequencies into contractions (for example, at 2 Hz the relaxation of 7 ± 3 % changed to a contraction of 24 ± 7 %). At the higher stimulation frequencies (8 - 32 Hz), L-NAME enhanced the contractions, although the increase only reached significance at 8 Hz (Figure 2.1B and 2.2A). At the frequencies 4 - 32 Hz, the contractions were followed by off-

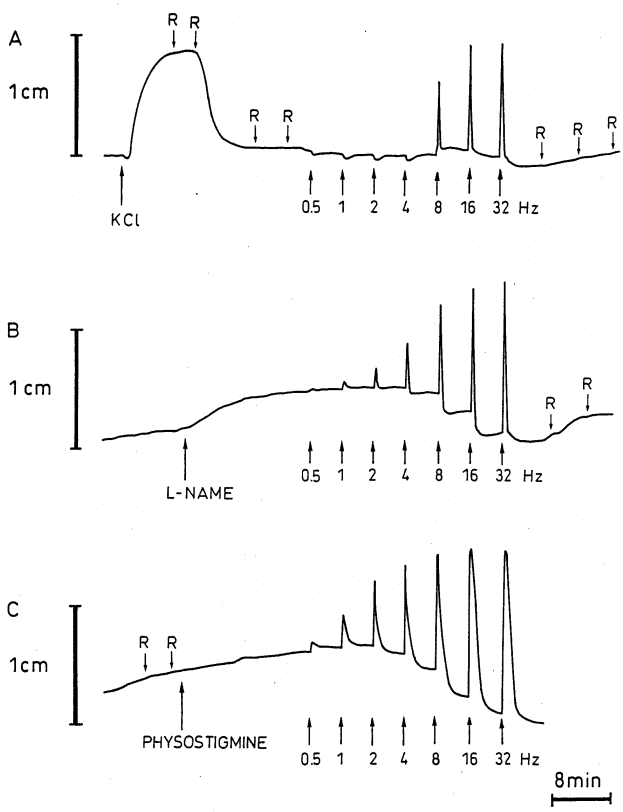

Figure 2.1 Representative traces from 1 tissue showing the responses to EFS (40 V, 0.5 ms, 0.5 - 32 Hz) with 10 s trains before (A) and after addition of $3x10^{-4}$ M L-NAME (B) and 10^{-6} M physostigmine in the continuous presence of L-NAME (C). KCl indicates the addition of 80 mM KCl, R indicates rinsing. Upon rinsing after the second trace, L-NAME was added again. During the incubation with L-NAME and physostigmine, the paper speed was reduced 2.5 fold.

relaxations, with a maximum at 16 Hz (Figure 2.2C). The less pronounced off-relaxation by stimulation at 32 Hz is probably due to the decreased tone level by the preceding off-relaxations. When physostigmine (10^{-6} M) was added to the medium already containing L-NAME, the tone further increased by 9 ± 4 % (n = 14). In the presence of L-NAME and physostigmine the contractions at the lowest stimulation frequencies further increased, and this was significant at 1 and 2 Hz. Also, after stimulation, tone declined at a slower rate than it had increased. In the presence of both substances, the contractions at all frequencies were significantly enhanced compared to responses in the absence of any drug (Figure 2.1C and 2.2A). The off-relaxations further increased, reaching significance at 2 and 4 Hz. At the stimulation frequencies 2 - 16 Hz the off-relaxations were significantly enhanced compared to off-relaxations in the absence of both drugs (Figure 2.2C) (n = 14 for all observations).

In a parallel set of tissues, the order of administration of physostigmine and L-NAME was reversed. The addition of physostigmine (10^{-6} M) increased the tone by 20 ± 5 % (n = 14). In the presence of physostigmine, contractions occurred at the frequencies tissues responded to EFS with relaxation in its absence, and the contractions at higher frequencies were significantly potentiated (Figure 2.2B); the off-relaxations were enhanced (Figure 2.2D). Addition of L-NAME ($3x10^{-4}$ M) to tissues already incubated with physostigmine increased tone by 54 ± 7 % (n = 14); this increase was more pronounced than when L-NAME was administered before adding physostigmine in the first series ($P < 0.01$). L-NAME had no additional effect on the electrically-induced responses compared to those in the presence of physostigmine alone (Figure 2.2B,D).

2.4.2. Influence of hexamethonium, atropine and TTX on the electrically-induced responses

Hexamethonium ($5x10^{-4}$ M), atropine (10^{-6} M) and TTX ($3x10^{-6}$ M) did not influence the basal tone in the three conditions tested. Hexamethonium had no influence on the responses to EFS in any of the conditions examined.

In medium containing guanethidine, atropine prevented the contractions at the higher stimulation frequencies and frequency-dependent relaxations occurred over the whole frequency range, except at 32 Hz where the relaxation amplitude decreased (Figure 2.3A). No off-relaxations occurred in the presence of atropine. In medium with guanethidine and L-NAME, EFS induced frequency-dependent contractions. In the presence of atropine, tissues responded with frequency-dependent relaxations at the stimulation frequencies 0.5 - 4 Hz

(Figure 2.3C). At the frequencies 8 - 32 Hz, the responses to EFS became very variable (contraction followed by relaxation, *n* = 3; relaxation, partial recovery of tone, sustained relaxation, *n* = 2; pure relaxation, *n* = 1). In medium with guanethidine, physostigmine and L-NAME, atropine abolished the contractile responses at 0.5 - 4 Hz, while the contractions at 8 - 32 Hz were significantly reduced (Figure 2.3E). In the presence of atropine, off-relaxations were significantly reduced at 8 Hz, equal at 16 Hz and significantly increased at 32 Hz.

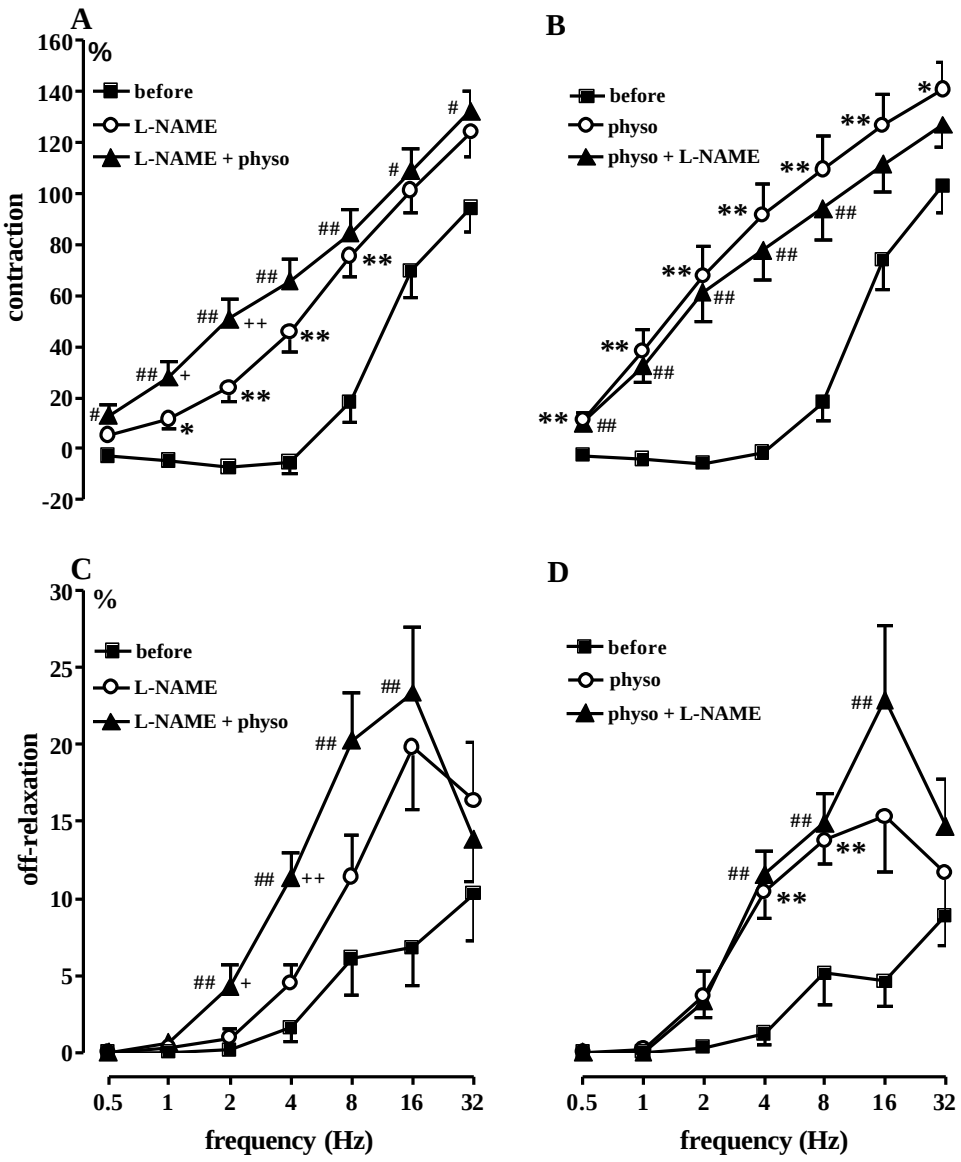

Figure 2.2 Mean ± s.e.mean (*n* = 14) frequency-response curves for the primary responses (A,B) and off-relaxations (C,D) to EFS (40 V, 0.5 ms, 0.5 - 32 Hz) with 10 s trains in circular muscle strips of the pig gastric fundus. Responses were obtained (A,C) in the absence and presence of $3x10^{-4}$ M L-NAME, and of $3x10^{-4}$ M L-NAME plus 10^{-6} M physostigmine; and (B,D) in the absence and presence of 10^{-6} M physostigmine, and of 10^{-6} M physostigmine plus $3x10^{-4}$ M L-NAME. The X-axis shows the frequency on a log-2-scale. * $P < 0.05$; ** $P < 0.01$: Significantly different from the response before addition of L-NAME (A) or physostigmine (B,D). + $P < 0.05$; ++ $P < 0.01$: Significantly different from the response in the presence of L-NAME (A,C). # $P < 0.05$; ## $P < 0.01$: Significantly different from the response in the absence of L-NAME and physostigmine (A - D).

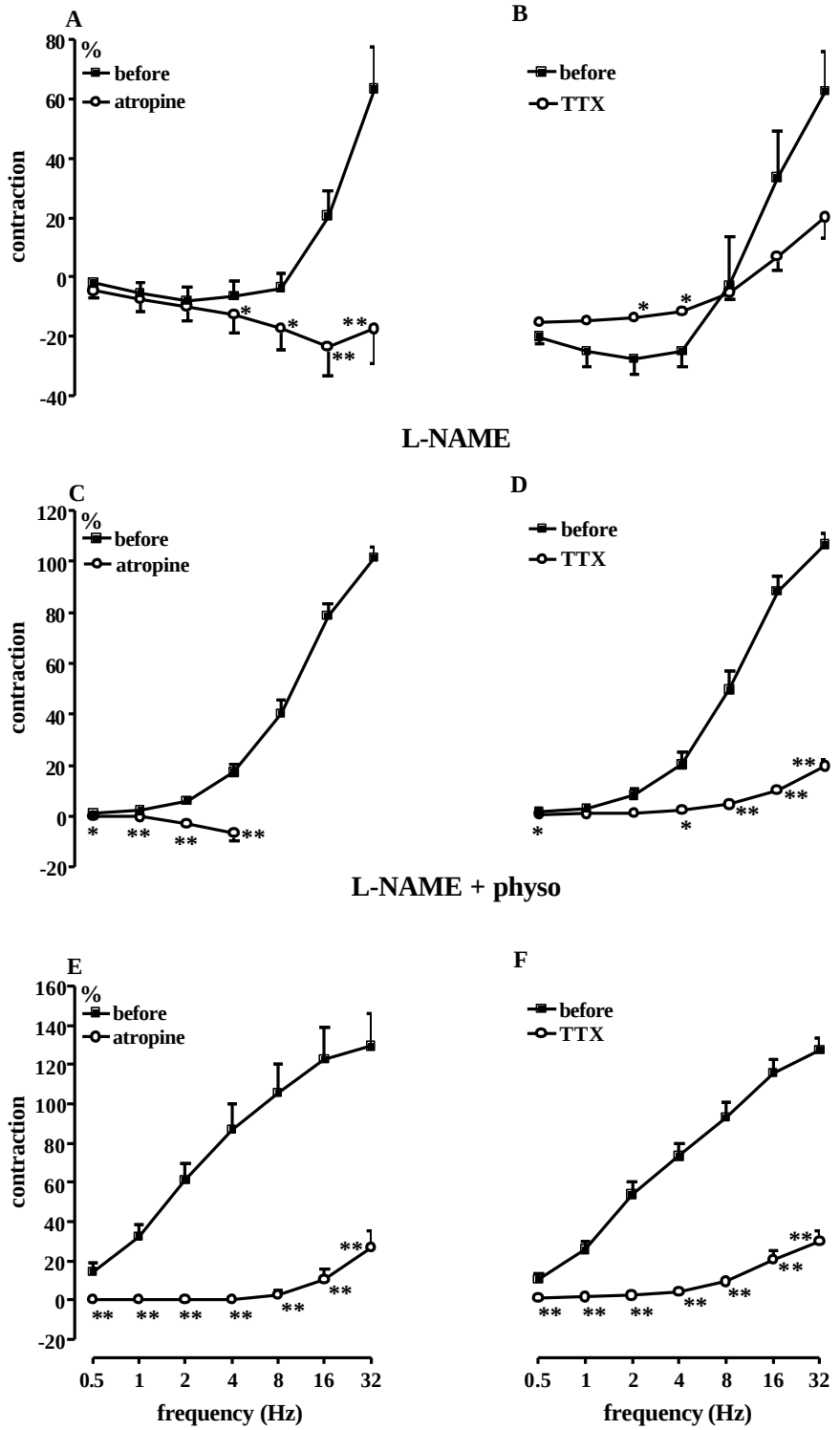

Figure 2.3 Mean ± s.e.mean frequency-response curves for the primary responses to EFS (40 V, 0.5 ms, 0.5 - 32 Hz) with 10 s trains in circular muscle strips of the pig gastric fundus. The medium contained $4x10^{-6}$ M guanethidine (A,B) (n = 6); $4x10^{-6}$ M guanethidine and $3x10^{-4}$ M L-NAME (C,D) (n = 6); $4x10^{-6}$ M guanethidine, $3x10^{-4}$ M L-NAME and 10^{-6} M physostigmine (E,F) (n = 8). Responses were obtained in the absence and presence of 10^{-6} M atropine (A,C,E) and in the absence and presence of $3x10^{-6}$ M TTX (B,D,F). In C, the responses at the frequencies 8 - 32 Hz in the presence of atropine became very complex and inconsistent and are described in the text. The X-axis shows the frequency on a log-2-scale. * P < 0.05; ** P < 0.01: Significantly different from the response before addition of atropine (A,C,E) or TTX (B,D,F).

In the three conditions tested, TTX nearly abolished the responses at the frequencies up to 4 Hz. However, at the higher frequencies, small contractions still occurred (Figure 2.3B,D and F). This was not due to the long incubation time of TTX before stimulation at 16 and 32 Hz was performed, as the TTX-resistant contractions were also observed in tissues only stimulated at 16 and 32 Hz (n = 4, results not shown).

2.4.3. Influence of α-chymotrypsin on off-relaxations

As described previously, off-relaxations were maintained in the presence of L-NAME, suggesting that they are not nitrergic in origin. To study if they were peptidergic, the influence of the peptidase α-chymotrypsin (10 U ml^{-1}) was studied on the off-relaxations in a medium containing $3x10^{-4}$ M L-NAME. In two parallel strips, one frequency-response curve was applied. When α-chymotrypsin was added, it induced a 51 ± 6 % (n = 8) increase in basal tone. To mimic this increase, 5-HT was added to parallel strips to cause a similar increase in tone (51 ± 7 %, n = 8). Off-relaxations in the presence of α-chymotrypsin were reduced at all frequencies applied, except at 32 Hz, compared with tissues contracted with 5-HT (3 ± 1, 8 ± 3, 19 ± 4, 26 ± 4 and 19 ± 5 % at 2, 4, 8, 16 and 32 Hz in tissues contracted with 5-HT, n = 8; 0, 3 ± 1, 8 ± 2 [$P < 0.05$], 15 ± 2 [$P < 0.05$] and 17 ± 3 % in the presence of α-chymotrypsin, n = 8).

2.4.4. Influence of L-NAME and atropine on electrically-induced responses in contracted tissues

Representative experiments of tissues contracted with 5-HT are shown in Figure 2.4. Addition to the bath medium of $3x10^{-7}$ M 5-HT induced stable contraction plateaus (57 ± 4 % of the maximal KCl-induced contraction, n = 24). Following rinsing, tone did not fully return to its original level. The contraction induced by the second and third administration of 5-HT attained 85 ± 6 % and 64 ± 6 % (n = 6), respectively, of the first contraction with 5-HT.

When EFS (40 V, 0.5 ms, 0.5 - 32 Hz, 10 s at 4 min intervals) was applied during the first contraction plateau, fast relaxations occurred at the stimulation frequencies 0.5 - 4 or 8 Hz (Figure 2.4A,D). Relaxations were larger than when tissues were not contracted (see Figure 2.1A). At the frequencies 16 and 32 Hz, frequency-dependent contractions consistently occurred (Figure 2.4A,D), and they were followed by off-relaxations.

Administration of L-NAME ($3x10^{-4}$ M) before the second administration of 5-HT increased resting tone by 28 ± 8 % (n = 6). L-NAME unmasked frequency-dependent contractions at the frequencies tissues responded to EFS with relaxation in its absence, and the contractions at higher frequencies were enhanced (Figure 2.4B). Contractions were followed by off-relaxations, already appearing at 0.5 Hz. When atropine (10^{-6} M) was added to the medium already containing L-NAME, tone was not further changed. Atropine completely abolished contractions: small relaxations at all frequencies, except at 32 Hz, occurred. At 32 Hz small contractions occurred, followed by off-relaxations (Figure 2.4C).

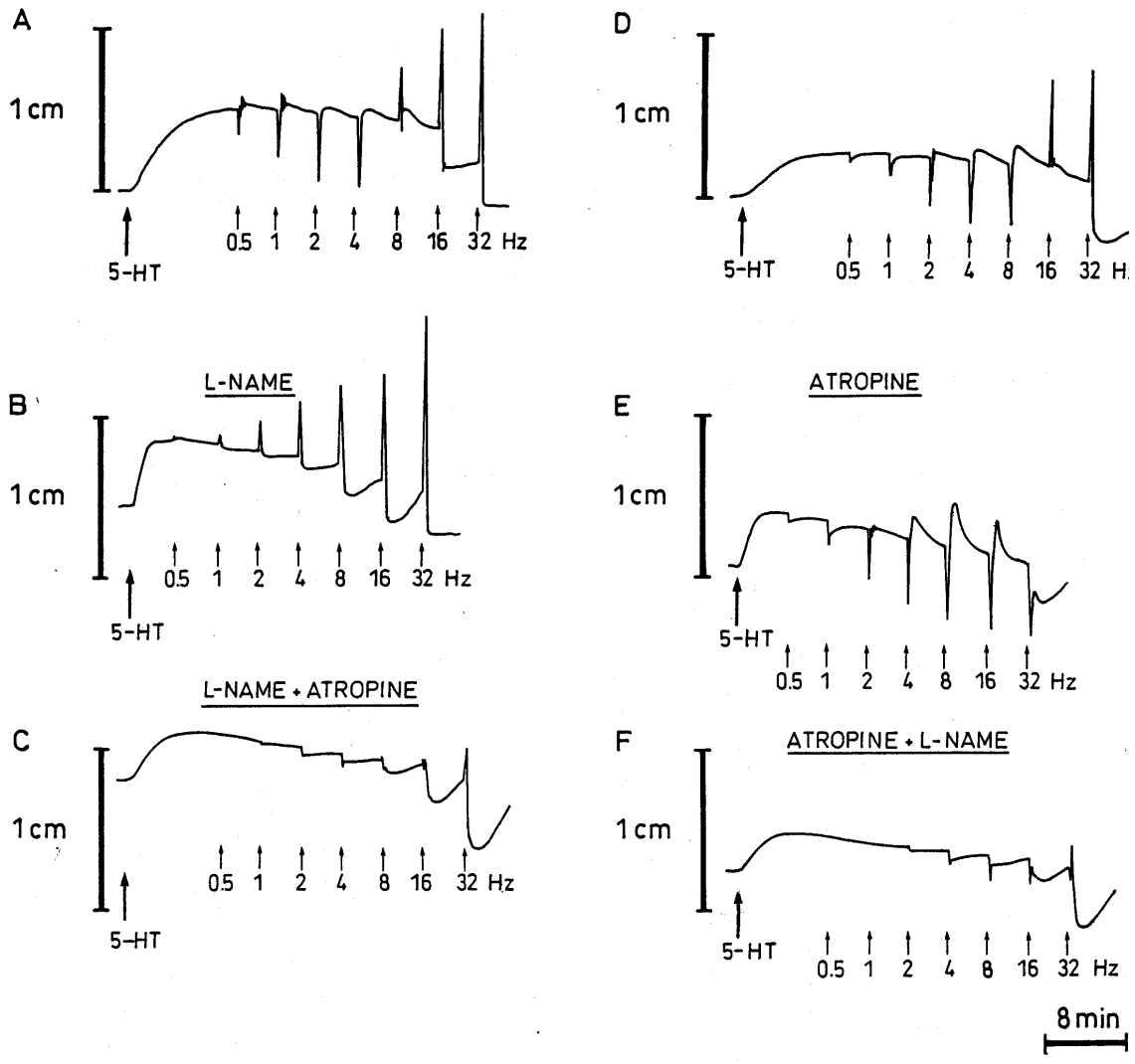

Figure 2.4 Representative traces from two tissues of the same animal showing the responses to EFS (40 V, 0.5 ms, 0.5 - 32 Hz) with 10 s trains when tissues were contracted by $3x10^{-7}$ M 5-HT. The responses in one tissue are shown before addition (A) and after addition of $3x10^{-4}$ M L-NAME (B), and of $3x10^{-4}$ M L-NAME plus 10^{-6} M atropine (C). In the second tissue, responses are shown before (D) and after addition of 10^{-6} M atropine (E), and of 10^{-6} M atropine plus $3x10^{-4}$ M L-NAME (F).

When the influence of atropine (10^{-6} M) was studied first, large frequency-dependent relaxations were obtained, followed by an off-contraction (Figure 2.4E). When L-NAME ($3x10^{-4}$ M) was administered to strips that previously received atropine, basal tone rose by 23 ± 5 % (n = 6), and the relaxations were reduced (Figure 2.4F); the responses were similar as when atropine was added after L-NAME (see Figure 2.4C).

When tissues were contracted by acetylcholine, added at a concentration to mimic the contraction amplitude caused by $3x10^{-7}$ M 5-HT, tissues reached a stable contraction plateau. Following rinsing, the resting tone was not completely regained. The contraction induced by the second addition of acetylcholine attained 77 ± 7 % of the first contraction by acetylcholine (n = 16). The response to EFS and the influence there upon of $3x10^{-4}$ M L-NAME was the same as when tissues were contracted with 5-HT (n = 8, results not shown).

2.4.5. Influence of hexamethonium and L-NAME on the contractions by exogenous acetylcholine

Administration of acetylcholine (10^{-9} - $3x10^{-4}$ M) induced sustained concentration-dependent contractions. In the presence of physostigmine, the concentration-response curve of acetylcholine was shifted to the left (EC_{50}: 2.8 ± 1.0 x 10^{-6} M in the absence and 6.5 ± 1.3 x 10^{-8} M in the presence of physostigmine; n = 12, $P < 0.01$).

In the absence of physostigmine, the contractions by acetylcholine were reproducible in control tissues. The responses were not influenced by $5x10^{-4}$ M hexamethonium (n = 4) nor by $3x10^{-4}$ M L-NAME, that increased basal tone by 26 ± 6 % (n = 6).

In the presence of physostigmine, the contractions by acetylcholine were not fully reproducible as the responses to $3x10^{-8}$ till $3x10^{-7}$ M acetylcholine were significantly reduced upon a second administration in the control tissues ($P < 0.05$; n = 6). L-NAME ($3x10^{-4}$ M) reduced the contractions significantly ($P < 0.01$) from $3x10^{-8}$ M acetylcholine onwards. The decrease of the maximal response to acetylcholine (from 112 ± 5 to 66 ± 8 %, n = 6) mimicked the increase in basal tone occurring after the addition of L-NAME (49 ± 4 %, n = 6).

2.4.6. Influence of SNP on electrically and acetylcholine-induced contractions

To study the influence of exogenous NO, the effect of 10^{-5} M SNP was investigated in medium containing $3x10^{-4}$ M L-NAME. The solvent of SNP, added after obtaining the initial responses to electrical stimulation at 4 Hz and to acetylcholine, had no effect on basal tone. When the tissues were stimulated 15 times at 4 Hz at 4 min intervals, the responses were decreased as compared to that obtained before the addition of solvent (66 ± 18 % at the first stimulation, 50 ± 20 % at the 15th stimulation, n = 7). The decrease was significant ($P < 0.05$) at the 14th and 15th stimulation. The responses to electrical stimulation in the presence of SNP depended upon the degree of relaxation induced by SNP. In three tissues out of seven, SNP decreased the basal tone by 56 ± 11 %. The contractile response to the first and second stimulation at 4 Hz after reaching the maximal relaxation by SNP, was significantly decreased to 41 ± 3 % and 65 ± 2 %, respectively, as compared to the response before addition of SNP; the contraction amplitude then progressively increased during the following stimulations and was no longer significantly different from the response before SNP. In the four other tissues, SNP decreased the basal tone by 113 ± 7 % and the response to the first till sixth stimulation at 4 Hz (162 ± 11, 234 ± 16, 266 ± 26, 296 ± 44, 313 ± 59 and 312 ± 64 %) was significantly increased in comparison to the response before SNP ($P < 0.05$ except for the 2nd and 3rd stimulation where $P < 0.01$).

In control tissues, the responses to the three additions of acetylcholine in the presence of the solvent of SNP were reproducible (Figure 2.5A). The responses to acetylcholine in the presence of SNP were again dependent on the degree of relaxation induced by SNP. In the tissues of three animals out of seven, SNP decreased the basal tone by 58 - 67 %. When acetylcholine was administered immediately when the maximal relaxation by SNP was reached, the response was significantly decreased to 23 ± 13 % ($P < 0.05$, unpaired t-test, n = 3; Figure 2.5B). When acetylcholine was administered 20 or 40 min after reaching the maximal relaxation by SNP, the amplitude of the contraction was not significantly different from that before addition of SNP (n = 3 for both; Figure 2.5C and D, respectively). In the tissues of four other animals, SNP decreased the tone by 106 - 118 %. In this case, the response to acetylcholine tended to be more pronounced than before administration of SNP: 151 ± 28 %, 191 ± 23 % ($P < 0.05$) and 222 ± 42 % at 0, 20 and 40 min, respectively, after reaching the maximal relaxation by SNP.

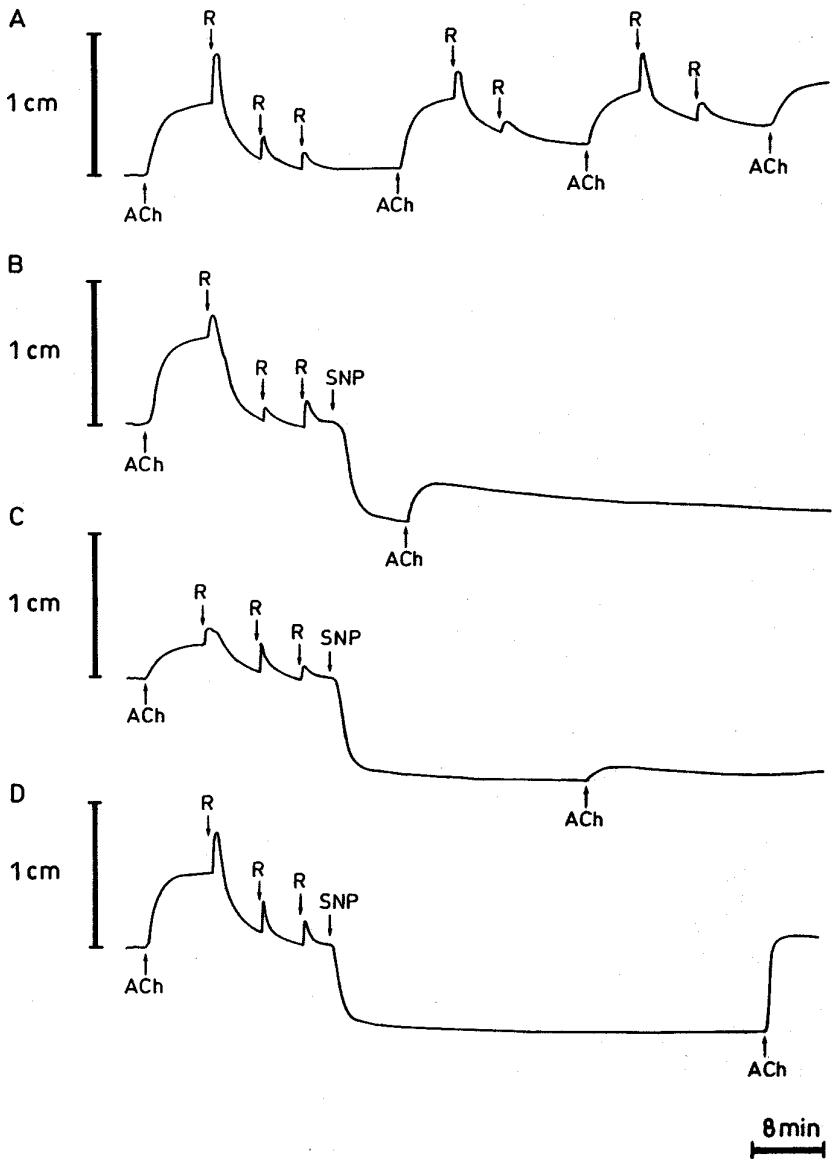

Figure 2.5 Representative traces from four tissues of the same animal showing the effect of acetylcholine (ACh) administered before and 0 (B), 20 (C) or 40 (D) min after the SNP-induced relaxation attained maximum. The solvent was added to the control tissue (A), which was contracted by acetylcholine at time points corresponding with the three other tissues. R indicates rinsing.

2.5. Discussion

The aim of this study was to investigate the interaction of the nitrergic and cholinergic innervation in the pig gastric fundus, by investigation of the responses to electrical field stimulation in the presence of guanethidine to exclude adrenergic influences. The responses studied were due to activation of postganglionic neurones, as the nicotinic receptor antagonist hexamethonium had no influence and the sodium channel blocker TTX abolished them except

for a small contractile response with stimulation at 16 and 32 Hz. At these frequencies, some direct smooth muscle cell activation might thus contribute to the responses. Alternatively, some transmitter release not involving conducted action potentials might occur at the nerve endings.

The inhibition of the relaxant responses by the NO synthase inhibitor L-NAME and of the contractile responses by the muscarinic antagonist atropine confirm that the principle neurotransmitters involved are the relaxant NO and the contractile acetylcholine (Miyazaki *et al.*, 1991; Lefebvre *et al.*, 1995). It has been suggested that L-NAME is able to antagonise muscarinic receptors (Buxton *et al.*, 1993) but the opposite effect of L-NAME *versus* atropine in the pig gastric fundus clearly illustrates that this is not the case in this tissue. Both transmitters are released at all stimulation frequencies, but the nitrergic contribution is dominant at the lower frequencies of stimulation, while the cholinergic contribution is dominant at the highest frequencies, corresponding to what has been observed in the opossum lower oesophageal sphincter (Cellek & Moncada, 1997). Upon concomitant release, NO and acetylcholine functionally antagonise each other. It has also been shown that NO is able to modulate cholinergic responses by prejunctional inhibition of acetylcholine release in the guinea-pig ileum (Wiklund *et al.*, 1993; Kilbinger & Wolf, 1994; Hebeiβ & Kilbinger, 1996), canine ileum (Hryhorenko *et al.*, 1994) and rat trachea (Sekizawa *et al.*, 1993). Two lines of results in the pig gastric fundus suggest that the potentiating effect of L-NAME on the electrically-induced contractions reflects only functional antagonism of acetylcholine by NO. First, the effect of L-NAME was studied on contractions by exogenous acetylcholine. The latter are related to activation of muscarinic receptors as hexamethonium had no influence; some degree of activation of neurones *via* ganglionic nicotinic receptors is thus excluded. In the absence of physostigmine, the acetylcholine-induced contractions were not influenced by L-NAME, excluding a non-specific potentiating effect of L-NAME on acetylcholine-induced contractions. The cholinesterase inhibitor physostigmine potentiated the contractions to acetylcholine as expected in view of the previously demonstrated presence of cholinesterase in the tissue (Miyazaki *et al.*; 1991). In the presence of physostigmine, L-NAME even reduced the responses to the higher concentrations of acetylcholine but this is probably related to the pronounced increase in tone by L-NAME in these conditions. Second, the influence of exogenous NO (SNP) was studied on electrically and acetylcholine-induced contractions. The influence of SNP on the responses was clearly dependent on the degree of relaxation induced by SNP as no inhibition of the cholinergic contractions was observed in tissues that showed a pronounced relaxation to SNP. However, in tissues moderately relaxing to SNP,

the cholinergic contractions were inhibited but the inhibition of the acetylcholine-induced responses was as pronounced as that of the electrically-induced ones and showed the same recuperation with time. In case of a prejunctional effect, a more pronounced inhibition of the electrically-induced contractions is expected. The conclusion that the interaction between NO and acetylcholine in the pig gastric fundus is only localized at the postjunctional level corresponds with what was shown in the opossum lower oesophageal sphincter (Cellek & Moncada, 1997), the guinea-pig gastric fundus (Milenov & Kalfin, 1996), the guinea-pig trachea (Brave *et al.*, 1991) and human airways (Ward *et al.*, 1993) and illustrates that the type of interaction depends on the species and tissue.

The augmentation of the electrically-induced contractions upon NO synthase inhibition with L-NAME was not maximal as the contractions were further increased by cholinesterase inhibition with physostigmine. However, in the presence of physostigmine, the contractile response at each frequency seems maximal as the further addition of L-NAME had no influence even at the lower frequencies where NO is predominant in the absence of inhibitors. Thus NO seems not to be able to counteract acetylcholine when the breakdown of the latter is inhibited. Alternatively, the pronounced increase in tone by administration of L-NAME in the presence of physostigmine might mask the potentiating effect of L-NAME in the presence of physostigmine. A final possibility is that acetylcholine also counteracts the nitrergic innervation at a prejunctional level. Inhibition of the breakdown of acetylcholine might then lead to a nearly complete prejunctional inhibition of NO release so that the addition of L-NAME has no influence. Prejunctional inhibition of nitrergic nerves *via* muscarinic receptors has been proposed in the rat anococcygeus (Li and Rand, 1989) and in monkey ciliary arteries (Toda *et al.*, 1998).

In rabbit gastric corpus, the cholinergic-nitrergic interaction can be influenced by the degree of contraction of the tissues and by the contractile agent used (Baccari *et al.*, 1997). In basal conditions, tissues responded to EFS (2 - 16 Hz) with contractions, that were enhanced by NO synthase inhibitors and abolished by atropine (Baccari *et al.*, 1993). When tone was increased by substance P or prostaglandin $F_{2\alpha}$, EFS induced relaxations, that were reversed into contractions by N^G-nitro-L-arginine (L-NOARG). However, in the presence of high concentrations of the muscarinic antagonists scopolamine or atropine, L-NOARG had no influence on the EFS-induced relaxations. When tone was increased by carbachol, the EFS-induced relaxations became progressively less sensitive to L-NOARG corresponding with the degree of contraction (Baccari *et al.*, 1997). These phenomena were not observed in the pig gastric fundus. When tissues were contracted by 5-HT and acetylcholine, the same pattern of

electrically-induced responses was observed as in basal conditions, i.e. relaxations at low frequencies (with increased amplitude as the relaxant effect of NO can become more manifest at increased tone) and contractions at the higher frequencies, and L-NAME reversed the relaxations into contractions. As tested upon contraction with 5-HT, L-NAME was able to almost abolish the relaxations in the presence of atropine. Thus, in the pig gastric fundus, the cholinergic-nitrergic interaction does not depend upon the degree of contraction and the contractile agent.

L-NAME consistently increased the tone of the tissues. We previously observed (Lefebvre *et al.*, 1995) that this effect is partially prevented by L-arginine, suggesting that it is related to suppression of NO synthesis and that a tonic nitrergic inhibition is present. Also physostigmine increased basal tone, suggesting that some tonic acetylcholine release is present. Apparently, the amount of acetylcholine when cholinesterase is not inhibited is not sufficient to contribute to the tone of the tissues as atropine has no influence. As TTX did not influence the tone of the tissues, tonic release of NO and acetylcholine *via* action potential conduction seems excluded but some leakage out of the nitrergic and cholinergic nerves might occur. L-NAME increased basal tone significantly more in the presence of physostigmine. In the porcine ileum, L-NOARG had no influence in basal conditions but it increased phasic activity dramatically in the presence of neostigmine (Fernández *et al.*, 1998). It was suggested that basal release of NO is poor but is strongly enhanced in the presence of significant levels of acetylcholine.

In the presence of atropine, only relaxations occurred at all stimulation frequencies, but in the concomitant presence of L-NAME small contractions sometimes occurred at the higher stimulation frequencies. This probably reflects the higher levels of acetylcholine overcoming the muscarinic receptor antagonism, when acetylcholine is no longer functionally antagonised by NO. This is also suggested by the further increase of these contractions in the presence of physostigmine. In the presence of atropine and L-NAME, small relaxations were obtained by electrical field stimulation, suggesting the possibility of release of another non-nitrergic inhibitory neurotransmitter. This was still more evident from the occurrence of the off-relaxations that were not prevented by L-NAME. The off-relaxations were potentiated by physostigmine and inhibited by atropine, suggesting that cholinergic activation is required to observe them. This might imply that the cholinergic contraction induces a rebound activation of inhibitory neurones. As the off-relaxations were partially reduced by the peptidase α-chymotrypsin, a peptide seems to be involved. A candidate is VIP, as double-labelling for NOS and VIP showed that both substances co-exist in a major part of the intrinsic nitrergic

neurones in the myentericus plexus (Lefebvre *et al.*, 1995). The contractile effect of α-chymotrypsin *per se*, that we had observed before in the absence of L-NAME (Lefebvre *et al.*, 1995) was also observed in this study in the presence of L-NAME, which itself increased the tone. A contractile effect of α-chymotrypsin has also been described in the rat gastric fundus (Gilfoil & Kelly, 1966) and most probably reflects a non-specific action and not cleavage of a continuously leaking inhibitory peptide. Even in the presence of L-NAME and α-chymotrypsin, some degree of off-relaxation was maintained. This might be related to penetration problems of α-chymotrypsin into the tissue or to a third non-peptide transmitter. In longitudinal muscle strips of the pig gastric fundus, ATP seems not involved (Ohga & Taneike, 1977).

In conclusion, our results show that endogenous NO is able to markedly interfere with cholinergic neurotransmission in the pig gastric fundus most probably by functional antagonism of acetylcholine at the level of the smooth muscle cells.

2.6. References

ABRAHAMSSON, H. (1986). Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch. Int. Pharmacodyn.*, **280** (Suppl.), 50 - 61

ANDREWS, P.L.R. (1990). Central organization of the vagal drive to the nonadrenergic, noncholinergic neurones controlling gastric motility. *Arch. Int. Pharmacodyn.*, **303**, 167 - 198

BACCARI, M.C., BERTINI, M. & CALAMAI, F. (1993). Effects of L-N^G-nitro arginine on cholinergic transmission in the gastric muscle of the rabbit. *Neuroreport*, **4**, 1102 - 1104

BACCARI, M.C., IACOVIELLO, C. & CALAMAI, F. (1997). Nitric oxide as modulator of cholinergic neurotransmission in gastric muscle of rabbits. *Am. J. Physiol.*, **273** (36), G456 - G463

BRAVE, S.R., HOBBS, A.J., GIBSON, A. & TUCKER, J.F. (1991). The influence of L-N^G-nitro-arginine on field stimulation induced contractions and acetylcholine release in guinea pig isolated tracheal smooth muscle. *Bioch. Bioph. Res. Comm.*, **179**, 1017 - 1022

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72**, 387 - 395

CELLEK, S. & MONCADA, S. (1997). Nitrergic modulation of cholinergic responses in the opossum lower oesophageal sphincter. *Br. J. Pharmacol.*, **122**, 1043 - 1046

D'AMATO, M., CURRO, D. & MONTUSCHI, P. (1992). Evidence for dual components in the non-adrenergic non-cholinergic relaxation in the rat gastric fundus: role of endogenous nitric oxide and vasoactive intestinal polypeptide. *J. Autonom. Nerv. Syst.*, **37**, 175 - 186

DESAI, K.M., WARNER, T.D., BISHOP, A.E., POLAK, J.M. & VANE, J.R. (1994). Nitric oxide, and not vasoactive intestinal peptide, as the main neurotransmitter of vagally induced relaxation of the guinea-pig stomach. *Br. J. Pharmacol.*, **113**, 1197 - 1202

FERNANDEZ, E., GUO, X., VERGARA, P. & JIMENEZ, M. (1998). Evidence supporting a role for ATP as non-adrenergic non-cholinergic inhibitory transmitter in the porcine ileum. *Life Sciences*, **62**, 15, 1303 - 1315

GILFOIL, T.M. & KELLY, C.A. (1966). Mechanism of action on plain muscle. *Br. J. Pharmacol. Chemother.*, **27**, 120 - 130

GRUNDY, D., GHARIB-NAXRI, M.K. & HUTSON, D. (1993). Role of nitric oxide and vasoactive polypeptide in vagally mediated relaxation of the gastric corpus in the anaesthetized ferret. *J. Auton. Nerv. Syst.*, **43**, 241 - 246

HEBEIβ, K. & KILBINGER, H. (1996). Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. *Br. J. Pharmacol.*, **118**, 2073 - 2078

HRYHORENKO, L.M., WOSKOWSKA, Z. & FOX-THRELKELD, J.-A. E.T. (1994). Nitric oxide (NO) inhibits release of acetylcholine from nerves of isolated circular muscle of the canine ileum: relationship to motility and release of nitric oxide. *J. Pharmacol. And Exp. Ther.*, **271**, 918 - 926

IVERSEN, H.H., CELSING, F., LEONE, A.M., GUSTAFSSON, L.E. & WIKLUND, N.P. (1997). Nerve-induced release of nitric oxide in the rabbit gastrointestinal tract as measured by in vivo microdialysis. *Br. J. Pharmacol.*, **120**, 702 - 706

KILBINGER, H. & WOLF, D. (1994). Increase by NO synthase inhibitors of acetylcholine release from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch Pharmacol.*, **349**, 543 - 545

LEFEBVRE, R.A. (1993). Non-adrenergic non-cholinergic neurotransmission in the proximal stomach. *Gen. Pharmacol.*, **24**, 257 - 266

LEFEBVRE, R.A., BAERT, E. & BARBIER, A.J. (1992a). Influence of N^G-nitro-L-Arginine on non-adrenergic non-cholinergic relaxation in the guinea-pig gastric fundus. *Br. J. Pharmacol.*, **106**, 173 - 179

LEFEBVRE, R.A., DE VRIESE, A. & SMITS, G.J.M. (1992b). Influence of vasoactive intestinal polypeptide and N^G-nitro-L-arginine methyl ester on cholinergic neurotransmission in the rat gastric fundus. *Eur. J. Pharmacol.*, **221**, 235 - 242

LEFEBVRE, R.A., SMITS, G.J.M. & TIMMERMANS, J.P. (1995). Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. *Br. J. Pharmacol.*, **116**, 2017 - 2026

LI, C.G. & RAND, M.J. (1989). Prejunctional inhibition of non-adrenergic non-cholinergic transmission in the rat anococcygeus muscle. *Eur. J. Pharmacol.*, **168**, 107 - 110

LI, C.G. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303 - 309

MILENOV, K. & KALFIN, R. (1996). Cholinergic-nitrergic interactions in the guinea-pig gastric fundus. *Neuropept.*, **30**, 365 - 371

MILLER, E.R. & ULLREY, D.E. (1987). The pig as a model for human nutrition. *Ann. Rev. Nutr.*, **7**, 361 - 382

MIYAZAKI, H., KOYAMA, I., NAKAMURA, H., TANEIKE, T. & OHGA, A. (1991). Regional differences in cholinergic innervation and drug sensitivity in the smooth muscles of the pig stomach. *J. Autonom. Pharmacol.*, **11**, 255 - 265

OHGA, A. & TANEIKE, T. (1977). Dissimilarity between the responses to adenosine triphosphate or its related compounds and non-adrenergic inhibitory nerve stimulation in the longitudinal smooth muscle of pig stomach. *Br. J. Pharmacol.*, **60**, 221 - 231

SEKIZAWA, K., FUKUSHIMA, T., IKARASHI, Y., MARUYAMA, Y. & SASAKI, H. (1993). The role of nitric oxide in cholinergic neurotransmission in rat trachea. *Br. J. Pharmacol.*, **110**, 816 - 820

TODA, N., TODA, M., AYAJIKI, K. & OKAMURA, T. (1998). Cholinergic nerve function in monkey ciliary arteries innervated by nitroxidergic nerve. *Am. J. Physiol.*, **274** (43), H1582 - H1589

WARD, J.K., BELVISI, M.G., FOX, A.J., MIURA, M., TADJKARIMI, S., YACOUB, M.H. AND BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. *J. Clin. Invest.*, **92**, 736 - 742

WIKLUND, C.U., OLGART, C., WIKLUND, N.P. & GUSTAFSSON, L.E. (1993). Modulation of cholinergic and substance P-like neurotransmission by nitric oxide in the guinea-pig ileum. *Br. J. Pharmacol.*, **110**, 833 - 839

CHAPTER 3

INFLUENCE OF NITRIC OXIDE DONORS AND OF THE a_2-AGONIST UK-14,304 ON ACETYLCHOLINE RELEASE IN THE PIG GASTRIC FUNDUS

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CHAPTER 3

INFLUENCE OF NITRIC OXIDE DONORS AND OF THE a_2-AGONIST UK-14,304 ON ACETYLCHOLINE RELEASE IN THE PIG GASTRIC FUNDUS

3.1. Summary

This study in circular muscle strips of the pig gastric fundus aimed to measure the release of acetylcholine directly and to investigate whether NO and α_2-adrenoceptor agonists can modulate acetylcholine release from cholinergic neurones. After incubation of the tissues with [^{3}H]-choline, basal and electrically induced release of tritium and [^{3}H]-acetylcholine was analysed in a medium containing physostigmine (10^{-5} M) as well as atropine (10^{-6} M). The NO synthase inhibitor L-N^G-nitroarginine methyl ester ($3x10^{-4}$ M), and the NO donors sodium nitroprusside (10^{-5} M) and 3-morpholino-sydnonimine (10^{-5} M) did not influence the basal release nor the electrically evoked release, indicating that NO does not modify [^{3}H]-acetylcholine release. The α_2-adrenoceptor agonist UK-14,304 (10^{-5} M) significantly inhibited the electrically evoked release of [^{3}H]-acetylcholine, and this effect was prevented by the α_2-adrenoceptor antagonist rauwolscine ($2x10^{-6}$ M), suggesting that presynaptic α_2-adrenoceptors are present on cholinergic neurones of the pig gastric fundus.

3.2. Introduction

The stomach is innervated by excitatory cholinergic neurones, by non-adrenergic non-cholinergic (NANC) inhibitory neurones, the latter being the final effectors of the vagally mediated gastric receptive relaxation (Abrahamsson, 1986) and by sympathetic noradrenergic fibers, whose cell bodies are localized in the celiac ganglion (Baumgarten, 1982). Nitric oxide (NO) has been proposed as an important inhibitory NANC neurotransmitter in the proximal part of the stomach (Li & Rand, 1990; Boeckxstaens *et al*, 1991; Desai *et al.*, 1991; Grundy *et al.*, 1994; Meulemans *et al*, 1995). In addition to its direct inhibitory effect on smooth muscle cells, NO might also decrease gastric motility by inhibiting the release of the excitatory neurotransmitter acetylcholine. In the guinea-pig ileum, NO donors decreased electrically induced acetylcholine release (Hebeiβ & Kilbinger, 1996), while NO synthase

inhibitors increased acetylcholine release in the guinea-pig and canine ileum (Kilbinger & Wolf, 1994 ; Hryhorenko *et al.*, 1994), suggesting that endogenous NO reduces acetylcholine release. A similar mechanism might occur at the level of the stomach, as electrically induced cholinergic contractions were enhanced by NO synthase inhibitors in smooth muscle strips of the rat and rabbit gastric fundus (Lefebvre *et al.*, 1992; Baccari *et al.*, 1993). The majority of noradrenergic fibers, innervating the gastrointestinal tract, end on intrinsic enteric neurones rather than on smooth muscle cells (Jacobowitz, 1965) and exert their effect by modulating the activity of the intrinsic neurones. In general, noradrenergic activity inhibits non-sphincteric gastrointestinal smooth muscle by inhibition of acetylcholine release from intrinsic excitatory cholinergic motor neurones *via* α_2-adrenoceptors (Furness & Costa, 1987; McIntyre & Thompson, 1992). This has also been shown at the level of the stomach; *in vivo*, stimulation of noradrenergic nerves supplying the cat stomach inhibited contractions induced by stimulation of the vagal cholinergic nerves (Jansson & Martinson, 1966; Jansson & Lisander, 1969) and *in vitro*, α_2- or α_2-like adrenoceptors have been shown to be present on postganglionic cholinergic neurones in the dog and rat gastric fundus (Lefebvre *et al.*, 1984; Verplanken *et al.*, 1984).

The pig is a good non-primate model for studying human digestive function in view of the similarity of the morphology and physiology of the gastrointestinal tracts (Miller & Ullrey, 1987). The pig gastric fundus is innervated by excitatory cholinergic, sympathetic noradrenergic and inhibitory NANC neurones (Ohga & Taneike, 1977; Miyazaki *et al.*, 1991) and NO is a major neurotransmitter of these NANC neurones (Lefebvre *et al.*, 1995). We recently studied the interaction between the cholinergic and nitrergic innervation in pig gastric fundus strips by measuring contractile activity; our results suggested that NO and acetylcholine functionally antagonise each other at the postjunctional level although prejunctional interaction could not be excluded (Leclere & Lefebvre, 1998). The aim of this study in the pig gastric fundus was to measure acetylcholine release directly and to investigate the influence of NO donors, NO synthase inhibition and stimulation of α_2-adrenoceptors on the electrically induced acetylcholine release.

3.3. Methods

3.3.1. Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the pig gastric fundus. The stomach was removed from healthy castrated male pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution (PSS). After the mucosa was removed, full thickness strips of approximately 1.5 cm in length and 0.3 cm in width (weight: 160 ± 5 mg; n = 92) were cut in the direction of the circular muscle. All strips were used the same day. Strips were mounted vertically between two platinum wire electrodes (40 mm x 0.5 mm) under a load of 2 g in 2 ml organ baths containing PSS (composition in mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose, 25 $NaHCO_3$, 0.0015 choline and 0.057 ascorbic acid), maintained at 37°C and gassed with carbogen (95% O_2 / 5% CO_2). Guanethidine ($4x10^{-6}$ M) was present in the medium throughout all experiments, unless otherwise stated. Electrical field stimulation (EFS) was applied by means of a stimulator (S88 Grass, USA).

3.3.2. Experimental protocol

During a 60 min equilibration period, the tissues were superfused at a rate of 2 ml/min, using a peristaltic pump (Gilson Minipuls, France). During the last 20 min the strips were subjected to continuous EFS (1 ms monophasic square wave pulses, 0.5 Hz, 40 V). After this equilibration period, superfusion was stopped and the preparations were incubated for 30 min with [^{3}H]-choline (5 µCi/ml) during which the tissues were stimulated electrically (40 V; 2 Hz; 1 ms) in order to label their cholinergic transmitter stores. In preliminary experiments, performed in the presence of 10^{-5} M physostigmine, it had been found that when frequencies of less than 1 Hz were applied during the incubation period with [^{3}H]-choline, no acetylcholine release could be detected; from 2 Hz onwards the percentage of acetylcholine formed reached its maximum.

After the labelling procedure, the strips were superfused (2 ml/min) for 90 min with PSS to remove loosely bound radioactivity. From now on the PSS contained in addition 10^{-5} M hemicholinium-3 to prevent the re-uptake of choline, and, unless otherwise stated, 10^{-5} M physostigmine to prevent the hydrolysis of acetylcholine and 10^{-6} M atropine to prevent the auto-inhibition of acetylcholine release.

After the washout period, the content of the organ bath (2 ml) was collected each 3 min. 1 ml of the samples was mixed with 4 ml of the scintillator containing solution Ultima Gold (Canberra Packard, USA). The strips were stimulated twice for 2 min (S_1 and S_2; 40 V, 1 ms, 4 Hz). S_1 started 13 min (5th sample), and S_2 79 min (27th sample) after the end of the washout period. Drugs were added 37 min (15th sample) before S_2, except for the α_2-adrenoceptor agonist, UK-14,304, which was added 4 min (26th sample) before S_2 (unless otherwise stated) and for tetrodotoxin (TTX), ω-conotoxin and calcium-free medium which were added or installed 34 min (16th sample) before S_2, and they remained present until the end of the experiment. At the end of the experiment, tissues were blotted and weighed.

3.3.3. Measurement of radioactivity and separation by HPLC of radioactive compounds

Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Canberra Packard, USA). External standardization was used to correct for counting efficiency. Electrical stimulation induced an increase in tritium overflow, in samples 5 up to 13 (for S_1) and 27 up to 35 (for S_2). The stimulation-induced increase in tritium overflow was calculated by subtracting basal tritium overflow. Basal tritium overflow during the period of enhanced tritium overflow was calculated by fitting a regression line through the values of the 4 samples just before stimulation and the values of the 4 samples starting from where overflow had returned to basal values after stimulation.

The amount of [^{3}H]-acetylcholine, [^{3}H]-choline and [^{3}H]-phosphorylcholine in the samples were analysed by reverse phase HPLC (Bischoff Chromatography, Germany; Hyperchrome-HPLC-column, 250 x 4.6 mm, prepacked with HYPERSIL – ODS 5.0 µm), as previously described (Wessler & Werhand, 1990). A 0.1 M phosphate buffer (pH 4.7) was used, containing methanol (8 vol %) and tetramethylammonium (0.2 mM). The flow was 0.5 ml/min and the effluent was collected in one min fractions. To estimate the retention times for each compound, the standards of [^{14}C]-acetylcholine, [^{14}C]-choline and [^{14}C]-phosphorylcholine were used (see Figure 3.1A and B).

HPLC was performed on 1 basal fraction before S_1 and S_2 (sample 3 before S_1 and sample 25 before S_2), and on the fraction with the highest radioactivity after stimulation (sample 5 and 27). 100 µl of the sample was injected into the HPLC; 27 fractions were collected, and they were mixed with 2.5 ml of Ultima Gold. Fractions 7 to 12 contained the peaks of phosphorylcholine and choline and were taken together to calculate the amount of phosphorylcholine and choline, as both peaks could not be separated completely with the

phosphate buffer we used. Fractions 15 to 25 were summed to calculate the amount of acetylcholine. The real amount of choline plus phosphorylcholine and of acetylcholine was calculated by subtracting the background counting. Background counting was calculated by fitting a regression line through the values of the first 5 fractions and fractions 26 and 27. Finally, the percentage of acetylcholine in each sample was calculated.

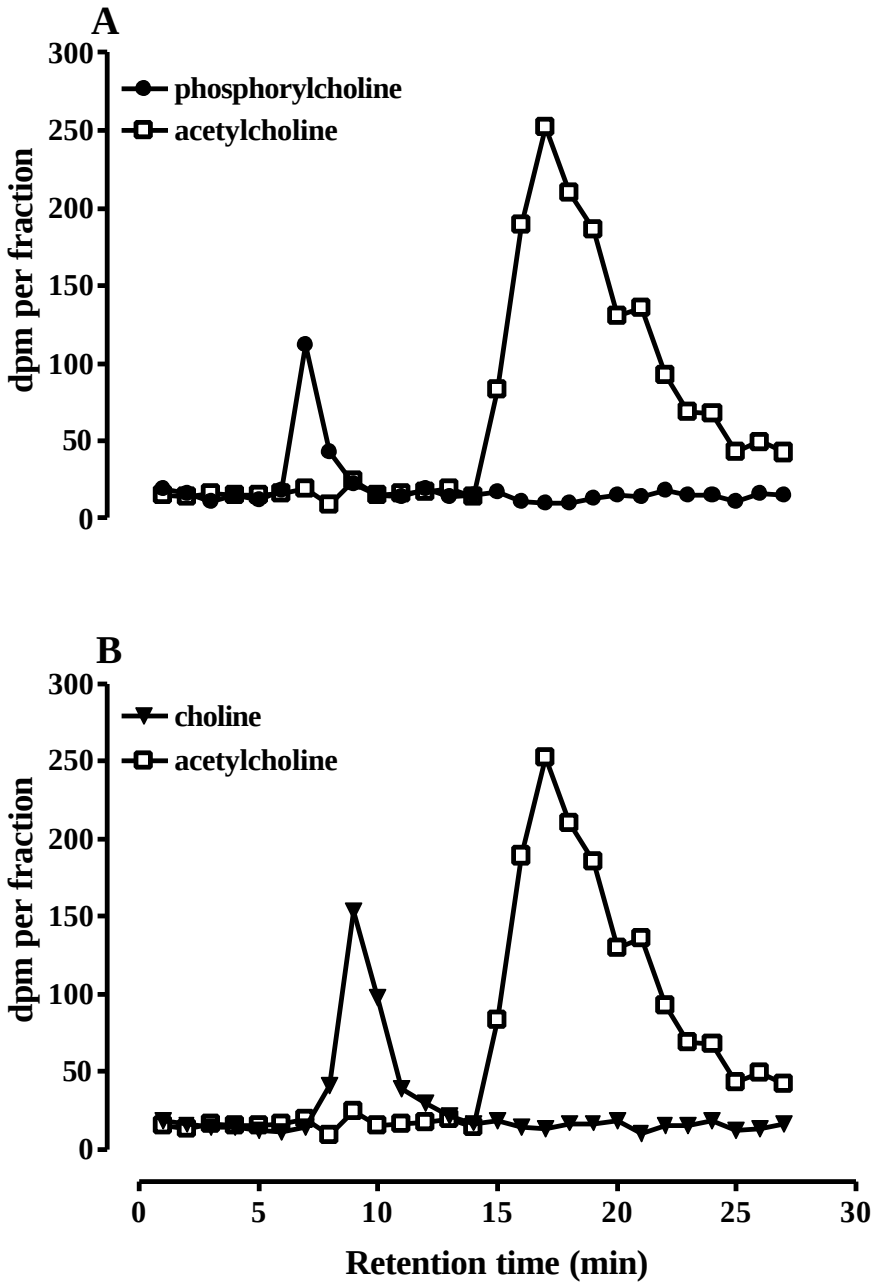

Figure 3.1 Separation of radiolabelled choline, acetylcholine and phosphorylcholine by reverse phase HPLC followed by liquid scintillation spectrometry. **A.** Radiochromatogram obtained after the injection of a solution that contained 125 dpm [^{14}C]-phosphorylcholine or 1200 dpm [^{14}C]-acetylcholine. **B.** Radiochromatogram obtained after the injection of a solution that contained 300 dpm [^{14}C]-choline or 1200 dpm [^{14}C]-acetylcholine. 100 µl of each solution was injected into the HPLC, and every min 500 µl was collected.

3.3.4. Drugs and radiochemicals

3-morpholino-sydnonimine (gift by Therabel), L-ascorbic acid, atropine sulphate, choline chloride, guanethidine sulphate, L-N^G-nitroarginine methyl ester and sodium nitroprusside were obtained from Sigma (St. Louis, USA), hemicholinium-3-bromide from RBI (USA), methanol from Lab-Scan (Dublin, Ireland), phentolamine mesilate from Novartis Pharma (Basel, Swiss), physostigmine salicylate from Federa (Brussels, Belgium), rauwolscine hydrochloride from Carl Roth KG (Karlsruhe, Germany), tetramethylammonium chloride from Merck-Schuchardt (Hohenbrunn, Germany), tetrodotoxin and ω-conotoxin-GVIA from Alomone labs (Jerusalem, Israel) and UK-14,304 tartrate (5-bromo-6-[-2-imidazolin-2-ylamino]-quinoxaline) (gift by Pfizer, Sandwich, England).

Radiochemicals: acetylcholine iodide [acetyl 1-^{14}C] (2.0 GBq/mmol) from American Radiolabeled Chemicals (ARC) (St. Louis, USA), [methyl-^{3}H]-choline chloride (2775 GBq/mmol) and [methyl-^{14}C]-choline chloride (2.0 GBq/mmol) from NEN (Boston, USA) and phosphoryl [methyl-^{14}C] choline, ammonium salt (2.07 GBq/mmol) from Amersham (Buckinghamshire, England).

Drugs were dissolved and diluted with distilled water. Stock solutions of 10^{-3} M TTX were kept frozen at –20°C and dilutions were made the day of the experiment.

3.3.5. Data analysis

The ratios S_2/S_1 for total radioactivity (TR) and for tritiated acetylcholine were calculated. Experimental data are expressed as means ± s.e.mean and *n* refers to the number of tissues. Results were compared by the unpaired *t*-test or by ANOVA followed by a *t*-test corrected for multiple comparisons (Bonferroni procedure) when more than two responses had to be compared. *P* values of less than 0.05 were considered statistically significant.

3.4. Results

3.4.1. Control experiments

Optimal incubation conditions were determined by evaluating tritium release induced by field stimulation at 40 V, 4 Hz, 1 ms for 2 min after incubation with ^{3}H-choline for 30 min. First, tissues were stimulated in PSS only containing guanethidine ($4x10^{-6}$ M) and hemicholinium-3 (10^{-5} M) (n = 4). A clearcut increase in total radioactivity (TR) followed the

stimulation, and 15 to 27 min were required after stimulation to re-establish the basal release of tritium. The response during the second stimulation was smaller, yielding a S_2/S_1 ratio for TR of 0.63 (Table 3.1). However, no acetylcholine was detected upon HPLC analysis, most probably due to the breakdown of acetylcholine by acetylcholinesterase. For this reason, a second set of experiments was performed in the presence of the acetylcholinesterase inhibitor physostigmine (10^{-5} M). The S_2/S_1 ratio for TR was lower than in the absence of the drug, although without reaching significance (n = 6; Table 3.1). However, HPLC now indicated the presence of acetylcholine in the samples upon stimulation with an S_2/S_1 ratio of 0.56 (Table 3.1). In the presence of physostigmine, the amount of TR released during S_1 (109 559 ± 10 550 dpm per g tissue; n = 6) tended to be lower in comparison with that in the absence of the drug (190 578 ± 59 819 dpm per g tissue; n = 4; $P > 0.05$). This might illustrate a more pronounced auto-inhibition of release by acetylcholine, that is less broken down in the presence of physostigmine. When atropine was present together with physostigmine, the amount of TR released during stimulation tended to be higher (249 560 ± 37 428 dpm per g tissue; n = 10) and the S_2/S_1 ratio for TR was 0.69. The ratio for acetylcholine was 1.08 (Table 3.1) and from now on, these conditions were used in further experiments.

The mean resting efflux of TR before S_1 (sample 3) in PSS containing guanethidine, hemicholinium-3, physostigmine and atropine was 20 540 ± 1 613 dpm per g tissue (n = 34). There was a progressive moderate decline in the basal efflux throughout the experiment (Figure 3.2A). The mean value of the resting efflux of TR before the second stimulation (sample 25), expressed in relation to that in sample 3 was 0.58 ± 0.02 (n = 34). The recovery of radioactivity by HPLC was complete for samples 3 and 25, containing basal efflux (99 ± 4 %; n = 34), while it was 91 ± 2 % (n = 34) for the samples obtained during stimulation (samples 5 and 27). For samples 3 and 25, 6 ± 2 % of TR was acetylcholine (n = 34), while this increased to 52 ± 2 % for samples during stimulation (n = 34). During stimulation (samples 5 and 27), there was a 2.5 ± 0.1-fold increase in the amount of [^{3}H]-phosphorylcholine and [^{3}H]-choline released compared to basal release ($P < 0.01$; n = 34; Figure 3.2B and C), while there was a 1 076 ± 386-fold increase in the amount of [^{3}H]-acetylcholine released compared to basal release ($P < 0.01$; n = 34; Figure 3.2B and C).

TTX ($3x10^{-6}$ M; n = 4), ω-conotoxin-GVIA (10^{-6} M; n = 3) or removal of extracellular calcium (n = 3) did not influence basal release of TR, but they nearly abolished the electrically-evoked tritium release as compared with control ($P < 0.01$). In control tissues (n = 4), the S_2/S_1 ratio for release of TR was 0.75 ± 0.03. After superfusion with TTX,

ω-conotoxin or calcium free medium, the S_2/S_1 ratios were respectively 0.07 ± 0.03, 0.07 ± 0.04 and 0.17 ± 0.06. Acetylcholine was not determined in these experiments.

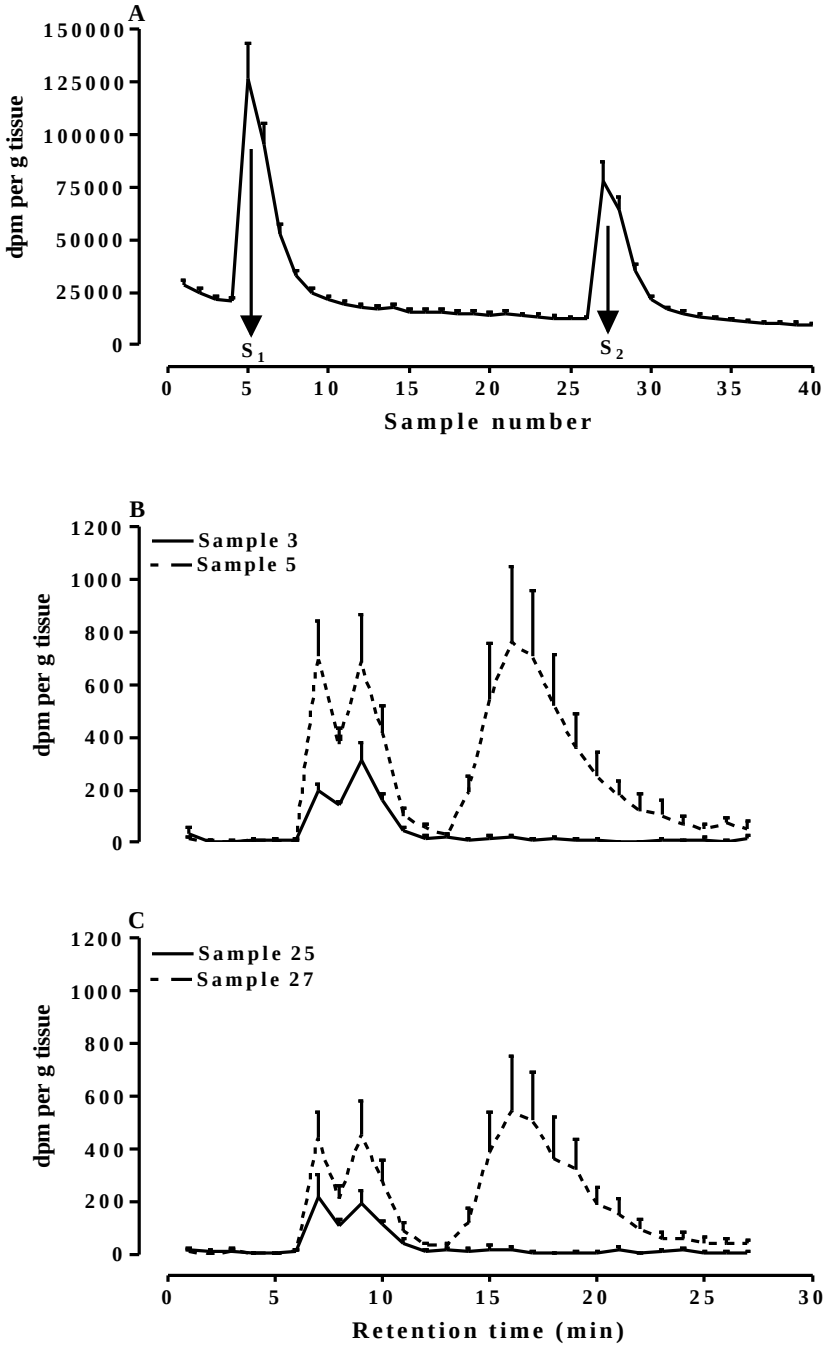

Figure 3.2 Influence of electrical field stimulation on the release of total radioactivity (TR) and [^{3}H]-acetylcholine from the pig gastric fundus. **A.** TR outflow from pig gastric fundus preparations preincubated with [^{3}H]-choline. The abscissa starts at the end of the washout period. Tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min), and the superfusate (2 ml) was collected in 3 min fractions. **B.** HPLC-separation of the radioactive outflow before (sample 3) and during (sample 5) S_1. **C.** HPLC-separation of the radioactive outflow before (sample 25) and during (sample 27) S_2. 100 µl of the samples were injected into the HPLC, and every min 500 µl was collected. For details about the peaks, see Figure 3.1. Results are given as mean ± s.e.mean (n = 34).

Table 3.1 Ratio (S_2/S_1) of the efflux of total radioactivity and [^{3}H]-acetylcholine induced by two periods of stimulation in different experimental conditions

Drugs present throughout experiment	TR	*n*	ACh	*n*
None	0.63 ± 0.04	4	n.d.	2
Physostigmine (10^{-5} M)	0.46 ± 0.05	6	0.56 ± 0.32	5
Physostigmine (10^{-5} M) + atropine (10^{-6} M)	0.69 ± 0.03*	10	1.08 ± 0.18	10

After incubation with [^{3}H]-choline and washout, tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min) with an interval of 66 min, and the superfusate (2 ml) was collected every 3 min. TR: total radioactivity; ACh: [^{3}H]-acetylcholine; n.d.: not detectable. * $P < 0.05$: Significantly different from strips where only physostigmine was present throughout the experiment.

3.4.2. The effects of a NO synthase inhibitor and NO donors

The NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, $3x10^{-4}$ M) did not influence basal release of TR (n = 6). When L-NAME was added before the second stimulation, neither the electrically evoked increase of TR released nor the release of acetylcholine were changed. In control tissues, the S_2/S_1 ratios for release of TR and acetylcholine were 0.72 ± 0.01 and 0.75 ± 0.03 respectively, while in the presence of L-NAME the ratios were 0.68 ± 0.06 and 0.69 ± 0.06 (n = 6).

The NO donors, sodium nitroprusside (SNP, 10^{-5} M; n = 6) and 3-morpholino-sydnonimine (SIN-1, 10^{-5} M; n = 6), did not affect basal release of TR or the electrically-evoked increase in release of TR or acetylcholine (Table 3.2).

3.4.3. The effects of UK-14,304 and α-adrenoceptor antagonists

The selective α_2-adrenoceptor agonist UK-14,304 (10^{-5} M), added 4 min before the second stimulation period, did not alter the basal efflux of TR. However, UK-14,304 significantly reduced the amount of TR and acetylcholine released upon electrical stimulation

Table 3.2 Influence of NO donors on the ratio (S_2/S_1) of the efflux of total radioactivity and [^{3}H]-acetylcholine induced by two periods of stimulation

Drugs added before S_2	TR	ACh
None	0.71 ± 0.04	0.66 ± 0.07
SNP (10^{-5} M)	0.79 ± 0.04	0.76 ± 0.04
None	0.71 ± 0.03	0.74 ± 0.05
SIN-1 (10^{-5} M)	0.75 ± 0.02	0.72 ± 0.09

After incubation with [^{3}H]-choline and washout, tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min) and the superfusate (2 ml) was collected every 3 min. Sodium nitroprusside (SNP, 10^{-5} M) and 3-morpholino sydnonimine (SIN-1, 10^{-5} M) were added 37 min before S_2. TR: total radioactivity; ACh: [^{3}H]-acetylcholine. Mean ± s.e.mean (n = 6).

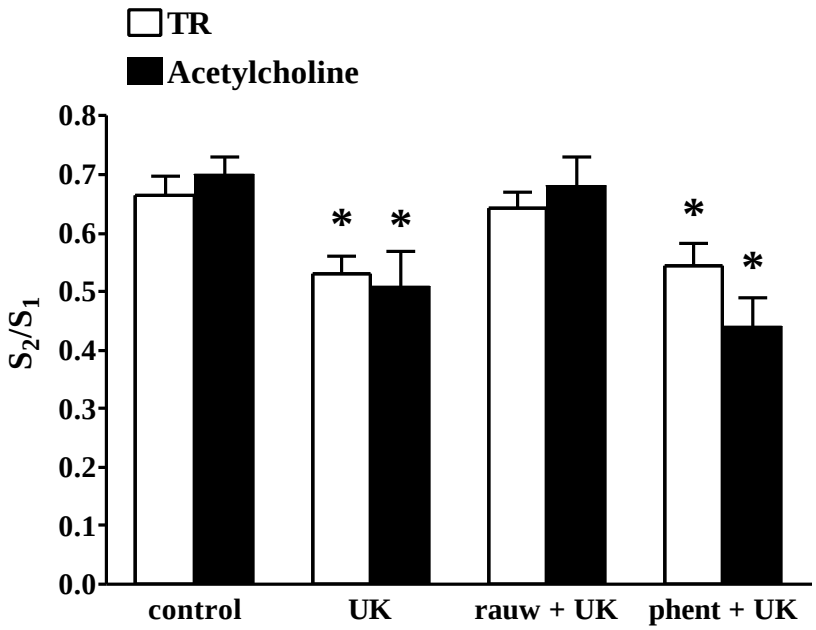

Figure 3.3 Effects of UK-14,304 (UK) and UK-14,304 in the presence of rauwolscine (rauw) or phentolamine (phent) on the electrically evoked release of total radioactivity (TR) and [^{3}H]-acetylcholine from preparations of pig gastric fundus preincubated with [^{3}H]-choline. Tissues were stimulated twice (S_1 and S_2, 40 V, 1 ms, 4 Hz, 2 min); rauwolscine ($2x10^{-6}$ M) and phentolamine (10^{-5} M) were added 37 min and UK-14,304 (10^{-5} M) 4 min before S_2. The electrical evoked efflux by S_2 is expressed as a ratio of that by S_1. Each column represents the mean ± s.e.mean (n = 6). * $P < 0.05$: Significantly different from control.

(n = 6; Figure 3.3). When the incubation time for UK-14,304 was increased to 37 min, the inhibition of the release of TR by UK-14,304 was not significantly increased compared to incubation during 4 min (S_2/S_1 ratios for TR: 0.70 ± 0.03 for control and 0.48 ± 0.04 for UK-14,304; $P < 0.01$; n = 5). The α_2-adrenoceptor antagonist rauwolscine ($2x10^{-6}$ M; n = 6) and the non-selective α-adrenoceptor antagonist phentolamine (10^{-5} M; n = 6) did not alter the basal efflux of TR. Rauwolscine prevented the inhibition of the electrically evoked release of TR and acetylcholine by UK-14,304 (Figure 3.3) while phentolamine was not able to counteract the effect of UK-14,304 on the amount of TR and acetylcholine released (Figure 3.3).

To study if endogenous noradrenaline is able to inhibit the release of acetylcholine *via* α_2-adrenoceptors, a series of experiments was done in the absence of guanethidine. In the absence of guanethidine, the mean basal release of TR before the first stimulation (sample 3) was 21 830 ± 2 655 dpm per g tissue (n = 6), which is not significantly different from the basal release in the presence of guanethidine. The increase in TR released after stimulation (204 520 ± 31 459 dpm per g tissue; n = 6) was also not significantly different from that in the presence of guanethidine (values in the presence of guanethidine: see control experiments). The S_2/S_1 ratios for TR and acetylcholine were 0.70 ± 0.02 and 0.71 ± 0.07 respectively for

control strips (n = 6). When rauwolscine ($2x10^{-6}$ M) was added between the two stimulation periods, the S_2/S_1 ratio for TR released was 0.71 ± 0.03 and that for acetylcholine was 0.75 ± 0.10 (n = 6).

3.5. Discussion

3.5.1. Control experiments

This study investigated the interaction of the nitrergic and sympathetic nerves with cholinergic transmission in the pig gastric fundus. Experiments were conducted on preparations which had been incubated with [^{3}H]-choline to incorporate [^{3}H]-acetylcholine into the cholinergic transmitter stores.

Separation of the samples with HPLC offers the possibility to separate on the one hand [^{3}H]-phosphorylcholine and [^{3}H]-choline, and on the other [^{3}H]-acetylcholine with a recovery rate of roughly 100 %. This is particularly interesting when only small amounts of acetylcholine are present. Because of the very small variation in the recovery rate (the s.e.mean on the recovery was maximally 4 %, see above), there is no need to correct every individual sample for the recovery rate by using an internal standard.

Incubation of the pig gastric fundus with [^{3}H]-choline resulted in the synthesis of [^{3}H]-acetylcholine in the nerve terminals, which was released by field stimulation. However, the synthesis of [^{3}H]-acetylcholine depended on the stimulation frequency during labelling. When frequencies of less than 1 Hz were used during labelling, as is done to label guinea-pig ileum and colon (see e.g. Szerb, 1975; 1976; Marcoli *et al.*, 1989; Wiklund *et al.*, 1993; Hebeiss & Kilbinger, 1998), no [^{3}H]-acetylcholine could be detected with HPLC, even in the presence of physostigmine. However, the amount of newly synthesised acetylcholine and the subsequent release of [^{3}H]-acetylcholine increased considerably when the stimulation frequency during labelling was increased from 1 to 4 Hz, and a maximum percentage of [^{3}H]-acetylcholine was reached if the tissue was stimulated during labelling at 2 Hz. This frequency-dependent labelling was also found in the myenteric plexus of the rat (Wessler & Werhand, 1990). The need for electrical stimulation during labelling suggests a low degree of spontaneous neuronal activity with a slow spontaneous exchange of newly-synthesised [^{3}H]-acetylcholine against unlabelled acetylcholine. This is confirmed by the observation that tetrodotoxin had no effect on basal efflux of TR and was also suggested in functional experiments were TTX did not influence the tone of the tissues (Leclere & Lefebvre, 1998). In contrast, in the guinea-pig

myenteric plexus large amounts of [^{3}H]-acetylcholine were synthesised even when the labelling period was carried out in the absence of electrical stimulation, suggesting a high degree of spontaneous neuronal activity as also demonstrated by the decrease in the basal release of acetylcholine with TTX (Szerb, 1975).

Field-stimulated release of TR was prevented by TTX or by the removal of extracellular calcium, indicating a neuronal release, as it is depending on, respectively, the opening of sodium channels, and the presence of calcium in the external medium. The calcium enters the cholinergic nerves *via* N-type calcium channels as ω-conotoxin-GVIA, a N-type calcium channel blocker, abolished the electrically evoked release of TR. [^{3}H]-choline and / or [^{3}H]-phosphorylcholine may be released by leakage from the nerve terminals as basal release was not influenced in the presence of these drugs.

Acetylcholine could only be detected when the acetylcholinesterase inhibitor physostigmine was present. In the presence of physostigmine, the stimulation-evoked outflow of TR tended to be lower than in its absence. Similarly, in the guinea-pig myenteric plexus physostigmine concentration-dependently depressed the release of TR (Kilbinger & Wessler, 1980). This might be due to an increase in the muscarinic inhibitory feedback, when the breakdown of acetylcholine is inhibited. This is indeed corroborated by the observation that the stimulation-evoked release of TR increased when the muscarinic antagonist atropine was present with physostigmine from the beginning of the experiments and that (results not shown) the ratio S_2/S_1 was significantly increased compared to control strips, when 10^{-6} M atropine was added between S_1 and S_2. The presence of muscarinic autoreceptors is in agreement with the current concept that presynaptic muscarinic receptors inhibit the release of acetylcholine from peripheral ends of parasympathetic nerve fibres in tissues of different species (see Starke *et al.*, 1989). As it was our aim to systematically analyse the amount of [^{3}H]-acetylcholine in the TR released, further experiments were performed in the presence of physostigmine and atropine. To determine the amount of [^{3}H]-acetylcholine released, the combination of a muscarinic antagonist and an acetylcholinesterase inhibitor was used in several studies (Wessler & Werhand, 1990; Wessler *et al.*, 1990; 1991); other authors, however, only used an acetylcholinesterase inhibitor (Hebeiß & Kilbinger, 1996; 1998; Hryhorenko *et al.*, 1994). This suggests that the degree of muscarinic auto-inhibition can differ from species to species and upon the experimental conditions.

Electrical stimulation caused, in addition to the release of [^{3}H]-acetylcholine, a moderate increase in the outflow of [^{3}H]-phosphorylcholine and [^{3}H]-choline, i.e. the stimulated increase in TR was not due to the release of [^{3}H]-acetylcholine alone. This is in

contrast with results in the guinea-pig and rat myenteric plexus, where electrical stimulation only caused an increase in [^{3}H]-acetylcholine release (Hebeiß & Kilbinger, 1996; Wessler & Werhand, 1990). [^{3}H]-phosphorylcholine can be formed *via* two pathways: 1) [^{3}H]-choline is not only a substrate for choline acetyltransferase to form acetylcholine, but also for choline-kinase to form [^{3}H]-phosphorylcholine which will be converted to [^{3}H]-cytidylyldiphosphocholine, which is further synthesised to [^{3}H]-phosphatidylcholine (Stryer, 1988); 2) [^{3}H]-phosphorylcholine can be liberated *via* the breakdown of [^{3}H]-phosphatidylcholine (Pelech & Vance, 1989). In canine ileum, transmural stimulation also caused an enhanced outflow of [^{3}H]-phosphorylcholine (Hryhorenko *et al.*, 1994). This also occurred in rat and guinea-pig trachea after field stimulation, however, after preganglionic stimulation of the parasympathetic nerves, the stimulated increase in tritium outflow was exclusively caused by an enhanced release of [^{3}H]-acetylcholine (Wessler *et al.*, 1990; 1991). As removal of the epithelium reduced the amount of [^{3}H]-phosphorylcholine liberated in response to field stimulation, Wessler *et al.* (1991) concluded that the electrical field injures epithelial cells with the subsequent liberation of [^{3}H]-phosphorylcholine from the outer leaflet of the cell membrane. As the mucosa was removed from the pig gastric fundus in our experiments, the source of the [^{3}H]-phosphorylcholine cannot be the epithelial cells. The increase in release of [^{3}H]-choline after electrical stimulation is probably due to the presence of [^{3}H]-choline in the vesicles after labelling, although we can not exclude that it is formed *via* breakdown of [^{3}H]-phosphorylcholine and [^{3}H]-phosphatidylcholine. However, as the S_2/S_1 ratio for [^{3}H]-acetylcholine was systematically similar to that for TR released, it will no longer be necessary to separate all radioactive components in future experiments as the results of TR reflect those of [^{3}H]-acetylcholine.

3.5.2. The effects of a NO synthase inhibitor and NO donors

We demonstrated before that blockade of NO synthase increased basal tone and that endogenous NO interferes with cholinergic neurotransmission in the pig gastric fundus, probably by functional antagonism of acetylcholine at the level of the smooth muscle cells (Leclere & Lefebvre, 1998). Many authors have reported that NO can also act presynaptically on cholinergic nerves to enhance the basal release of acetylcholine and to inhibit the electrically evoked release of acetylcholine (see review Kilbinger, 1996). As it is not possible to determine with certainty the site(s) of action of NO (i.e. pre- vs. postsynaptic) from functional experiments, the effects of a NO synthase inhibitor and NO donors on

[^{3}H]-acetylcholine release in the pig gastric fundus were now determined. Blockade of NO synthase with L-NAME, or addition of NO donors, in concentrations shown in our lab to induce maximal relaxation of circular muscle strips of the pig gastric fundus, did not significantly affect basal release of TR or electrically-evoked overflow of TR and [^{3}H]-acetylcholine, suggesting that NO does not enhance basal acetylcholine release or suppress electrically-evoked release of acetylcholine in the pig gastric fundus. This is in agreement with the findings of others who reported that NO donors and NO synthase inhibitors do not modify [^{3}H]-acetylcholine release in either tracheal or intestinal preparations (Brave et al, 1991; Ward et al., 1993; Ward et al, 1996; Milenov & Kalfin, 1996; Rae et al., 1998). Still, in view of the circular direction of the strips, it cannot be excluded that acetylcholine release from longitudinally directed interneurones and / or sensory neurones is not fully assessed; modulation of acetylcholine released by NO might still be present at this level.

3.5.3. The effects of UK-14,304 and α-adrenoceptor antagonists

Several investigators have reported that presynaptic inhibitory α_2-adrenoceptors are present on cholinergic nerves in various gastrointestinal tissues and in other tissues (see review: De Ponti *et al.*, 1996 and introduction). In this study, the α_2-adrenoceptor agonist UK-14,304 significantly reduced the stimulation-induced efflux of TR and [^{3}H]-acetylcholine in the pig gastric fundus. The reduction was smaller than in other species, where the depression of release with 10^{-5} M UK-14,304 was about 50 % or more (e.g. Wessler *et al.*, 1987). This was not due to the short incubation time of UK-14,304 as increasing the incubation time to 37 min did not increase the degree of inhibition of TR release by UK-14,304. The inhibition of the stimulated overflow produced by UK-14,304 was completely antagonised by rauwolscine, a selective α_2-adrenoceptor antagonist, indicating that cholinergic nerves of the pig gastric fundus are also endowed with α_2-adrenoceptors, causing inhibition of transmitter acetylcholine release. We have no explanation why phentolamine, a non-selective α-adrenoceptor antagonist, did not counteract the inhibitory action of UK-14,304. To study whether endogenous noradrenaline is able to inhibit acetylcholine release *via* the presynaptic α_2-adrenoceptors on the cholinergic neurones, experiments were performed in the absence of guanethidine. Both basal and electrically-evoked release were not lower compared to the release in the presence of guanethidine, suggesting that in pig gastric fundus sympathetic nerves do not modulate the release of acetylcholine *via* the

inhibitory α_2-adrenoceptors within the experimental conditions and / or that electrical field stimulation does not lead to the release of noradrenaline. This was confirmed when rauwolscine was added between S_1 and S_2; it was without effect on the electrically-evoked release of TR and [^{3}H]-acetylcholine while an increase is expected if endogenous noradrenaline were inhibiting acetylcholine release *via* the presynaptic α_2-adrenoceptors. Also in the guinea-pig ileum and rat trachea (Alberts & Stjärne, 1982; Fabiani *et al.*, 1997), no evidence to suggest that endogenous noradrenaline influences acetylcholine release was obtained.

In conclusion, the data provided by this study indicate that measurement of tritium release after incubation with [^{3}H]-choline can be used for measurement of endogenous acetylcholine output in response to cholinergic neuron stimulation in the pig gastric fundus. The method allowed to suggest the presence of presynaptic muscarinic and α_2-adrenoceptors on the cholinergic neurones. No evidence for the modulation of acetylcholine release by NO was obtained.

3.6. References

ABRAHAMSSON, H. (1986). Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch. Int. Pharmacodyn.*, **280** (Suppl.), 50 - 61

ALBERTS, P. & STJÄRNE, L. (1982). Facilitation, and muscarinic and α-adrenergic inhibition of the secretion of ^{3}H-acetylcholine and ^{3}H-noradrenaline from guinea-pig ileum myenteric nerve terminals. *Acta Physiol. Scand.*, **116**, 83 - 92

BACCARI, M.C., BERTINI, M. & CALAMAI, F. (1993). Effects of L-N^G-nitro arginine on cholinergic transmission in the gastric muscle of the rabbit. *Neuroreport*, **4**, 1102 - 1104

BAUMGARTEN, H.G. (1982). Morphological basis of gastrointestinal motility: structure and innervation of gastrointestinal tract. In: Bertaccini, G. (Ed), Gastrointestinal motility, Springer Verlag, Berlin, pp. 7 – 54

BOECKXSTAENS, G.E., PELCKMANS, P.A., BOGERS, J.J., BULT, H., DE MAN, J.G., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Release of nitric oxide upon stimulation of nonadrenergic noncholinergic nerves in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **256**, 441 – 447

BRAVE, S.R., HOBBS, A.J., GIBSON, A. & TUCKER, J.J. (1991). The influence of L-N^G-nitro-arginine on field stimulation induced contractions and acetylcholine release in guinea-pig isolated tracheal smooth muscle. *Biochem. Biophys. Res. Comm*., **179**, 1017 - 1022

D'AMATO, M., CURRO, D. & MONTUSCHI, P. (1992). Evidence for dual components in the non-adrenergic non-cholinergic relaxation in the rat gastric fundus: role of endogenous nitric oxide and vasoactive intestinal polypeptide. *J. Auton. Nerv. Syst*., **37**, 175 - 186

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.*, **69**, 59 - 78

DESAI, K.M., SESSA, W.C. & VANE, J.R. (1991). Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature*, **351**, 477 - 479

FABIANI, M.E., DINH, D.T. & STORY, D.F. (1997). Interaction of the renin-angiotensin system, bradykinin and sympathetic nerves with cholinergic transmission in the rat isolated trachea. *Br. J. Pharmacol*., **122**, 1089 - 1098

FURNESS, J.B. & COSTA, M. (1987). The enteric nervous system. Edinburgh: Churchill Livingstone

GRUNDY, D., GHARIB-NASERI, M.K. & HUTSON, D. (1993). Role of nitric oxide and vasoactive intestinal polypeptide in vagally mediated relaxation of the gastric corpus in the anaesthetized ferret. *J. Auton. Nerv. Syst*., **43**, 241 - 246

HEBEIβ, K. & KILBINGER, H. (1996). Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. *Br. J. Pharmacol*., **118**, 2073 - 2078

HEBEIβ, K. & KILBINGER, H. (1998). Nitric oxide-sensitive guanylyl cyclase inhibits acetylcholine release and excitatory motor transmission in the guinea-pig ileum. *Neuroscience*, **82**, 623 - 629

HRYHORENKO, L.M., WOSKOWSKA, Z. & FOX-THRELKELD, J.-A.E.T. (1994). Nitric oxide (NO) inhibits release of acetylcholine from nerves of isolated circular muscle of the canine ileum: relationship to motility and release of nitric oxide. *J. Pharmacol. Exp. Ther.*, **271**, 918 - 926

JACOBOWITZ, D. (1965). Histochemical studies of the autonomic innervation of the gut. *J. Pharmacol. Exp. Ther.*, **149**, 358 – 364

JANSSON, G. & LISANDER, B. (1969). On adrenergic influence on gastric motility in chronically vagotomized cats. *Acta Physiol. Scand.*, **76**, 463 - 471

JANSSON, G. & MARTINSON, J. (1966). Studies on the ganglionic site of action of sympathetic outflow to the stomach. *Acta Physiol. Scand.*, **68**, 184 - 192

KILBINGER, H. (1996). Modulation of acetylcholine release by nitric oxide. *Progress in Brain Research.*, **109**, 219 - 224

KILBINGER, H. & WESSLER, I. (1980). Inhibition by acetylcholine of the stimulation-evoked release of [^{3}H]acetylcholine from the guinea-pig myenteric plexus. *Neuroscience*, **5**, 1331 - 1340

KILBINGER, H. & WOLF, D. (1994). Increase by NO synthase inhibitors of acetylcholine release from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349**, 543 - 545

LECLERE, P.G. & LEFEBVRE, R.A. (1998). Investigation of the interaction between cholinergic and nitrergic neurotransmission in the pig gastric fundus. *Br. J. Pharmacol.*, **125**, 1779 - 1787

LECLERE, P.G. & LEFEBVRE, R.A. (1999). Nitrergic-cholinergic interaction in the pig stomach. *Fundam. Clin. Pharmacol.*, **13**, 517

LEFEBVRE, R.A., DE VRIESE, A. & SMITS, G.J.M. (1992). Influence of vasoactive intestinal polypeptide and N^G-nitro-L-arginine methyl ester on cholinergic neurotransmitters in the rat gastric fundus. *Eur. J. Pharmacol.*, **221**, 235 - 242

LEFEBVRE, R.A., SMITS, G.J.M. & TIMMERMANS, J.P. (1995). Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. *Br. J. Pharmacol.*, **116**, 2017 - 2026

LEFEBVRE, R.A., WILLEMS, J.L. & BOGAERT, M.G. (1984). Inhibitory effect of dopamine on canine gastric fundus. *Naunyn-Schmiedebergs Arch Pharmacol.*, **326**, 22 – 28

LI, C.G. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303 - 309

MARCOLI, M., DE PONTI, F., LECCHINI, S., CREMA, A. & FRIGO, G.M. (1989). [^{3}H]Acetylcholine release from the guinea-pig distal colon: comparison with ileal [^{3}H]acetylcholine release and effect of adrenoceptor stimulation. *J. Pharm. Pharmacol.*, **41**, 824 - 828

MCINTYRE, A.S. & THOMPSON, D.G. (1992). Review article: adrenergic control of motor and secretory function in the gastrointestinal tract. *Aliment. Pharmacol. Ther.*, **6**, 125 - 142

MEULEMANS, A.L., EELEN, J.G. & SCHUURKES, J.A. (1995). NO mediates gastric relaxation after brief vagal stimulation in anesthetized dogs. *Am. J. Physiol.*, **269**, G255 - G261

MILENOV, K. & KALFIN, R. (1996). Cholinergic-nitrergic interactions in the guinea-pig gastric fundus. *Neuropeptides*, **30**, 365 - 371

MILLER, E.R. & ULLREY, D.E. (1987). The pig as a model for human nutrition. *Ann. Rev. Nutr.*, **7**, 361 - 382

MIYAZAKI, H., KOYAMA, I., NAKAMURA, H., TANEIKE, T. & OHGA, A. (1991). Regional differences in cholinergic innervation and drug sensitivity in the smooth muscles of the pig stomach. *J. Autonom. Pharmacol.*, **11**, 255 - 265

OHGA, A. & TANEIKE, T. (1977). Dissimilarity between the responses to adenosine triphosphate or its related compounds and non-adrenergic inhibitory nerve stimulation in the longitudinal smooth muscle of pig stomach. *Br. J. Pharmacol.*, **60**, 221 - 231

PELECH, S.L. & VANCE, D.E. (1989). Signal transduction via phosphatidylcholine cycles. *Trends Biochem. Sci.*, **14**, 28 - 30

RAE, M.G., KHOYI, M.A. & KEEF, K.D. (1998). Modulation of cholinergic neuromuscular transmission by nitric oxide in canine colonic circular smooth muscle. *Am. J. Physiol.*, **275**, G1324 - G1332

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of transmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 864 - 989

STRYER, L. (1988). Biosynthesis of membrane lipids and steroid hormones. In: Biochemistry, Third edition. W.H. Freeman and company, New York, pp.547 - 574

SZERB, J.C. (1975). Endogenous acetylcholine release and labelled acetylcholine formation from [^{3}H]choline in the myenteric plexus of the guinea-pig ileum. *Can. J. Physiol. Pharmacol.*, **53**, 566 - 574

SZERB, J.C. (1976). Storage and release of labelled acetylcholine in the myenteric plexus of the guinea-pig ileum. *Can J. Physiol. Pharmacol.*, **54**, 12 - 22

VERPLANKEN, P.A., LEFEBVRE, R.A. & BOGAERT,M.G. (1984). Pharmacological characterization of *alpha* adrenoceptors in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **231**, 404 - 410

WARD, J.K., BELVISI, M.G., FOX, A.J., MIURA, M., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. *J. Clin. Invest.*, **92**, 736 - 743

WARD, S.M., DALZIEL, H.H., KHOYI, M.A., WESTFALL, A.S., SANDERS, K.M. & WESTFALL, D.P. (1996). Hyperpolarization and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli. *Br. J. Pharmacol.*, **118**, 49 - 56

WESSLER, I., ESCHENBRUCH, V., HALIM, S. & KILBINGER, H (1987). Presynaptic effects of scopolamine, oxotremorine, noradrenaline and morphine on [^{3}H]acetylcholine release from the myenteric plexus at different stimulation frequencies and calcium concentrations. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335**, 597 - 604

WESSLER, I., HELLWIG, D. & RACKÉ, K. (1990). Epithelium-derived inhibition of [^{3}H]acetylcholine release from the isolated guinea-pig trachea. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **342**, 387 - 393

WESSLER, I., KLEIN, A., POHAN, D., MACLAGAN, J. & RACKÉ, K. (1991). Release of [^{3}H]acetylcholine from the isolated rat or guinea-pig trachea evoked by preganglionic nerve stimulation; a comparison with transmural stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **344**, 403 - 411

WESSLER, I. & WERHAND, J. (1990). Evaluation by reverse phase HPLC of [^{3}H]acetylcholine release evoked from the myenteric plexus of the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 510 - 516

WIKLUND, C.U., OLGART, C., WIKLUND, N.P. & GUSTAFSSON, L.E. (1993). Modulation of cholinergic and substance P-like neurotransmission by nitric oxide in the guinea-pig ileum. *Br. J. Pharmacol.*, **110**, 833 - 839

CHAPTER 4

CHARACTERISATION OF PRE- AND POSTSYNAPTIC MUSCARINIC RECEPTORS IN CIRCULAR MUSCLE OF PIG GASTRIC FUNDUS

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CHAPTER 4

CHARACTERISATION OF PRE- AND POSTSYNAPTIC MUSCARINIC RECEPTORS IN CIRCULAR MUSCLE OF PIG GASTRIC FUNDUS

4.1. Summary

This study investigated the subtype of muscarinic receptors on the cholinergic neurones and smooth muscle in the circular muscle of the pig gastric fundus. Muscarinic antagonists, except MT-3, concentration-dependently inhibited the contractions induced by a given concentration of acetylcholine. Concentration-response curves by acetylcholine were shifted rightwards in a parallel manner without depression of the maximum by the muscarinic antagonists, except by MT-3 that induced a leftward shift. Correlation of the pIC_{50} and pA_2 values with published pK_i values for the five muscarinic receptor subtypes suggests that the muscarinic receptors on pig gastric fundus circular muscle belong to the M_3 subtype. Electrically-evoked contractions (40 V, 4 Hz, 0.25 ms, 2 min) were concentration-dependently inhibited by the muscarinic antagonists except for methoctramine and AF-DX 116, that increased the amplitude of the electrically-induced contractions in lower concentrations. MT-3 tended to increase the electrically-induced contractions. The antagonists, except MT-3, concentration-dependently increased the electrically-induced tritium outflow (40 V, 4 Hz, 0.25 ms, 2 min) after incubation of the tissues with [^{3}H]-choline. MT-3 ($3x10^{-8}$ and 10^{-7} M) decreased the electrically-induced tritium release. Correlation of the pIC_{50} values with published pK_i values for the different muscarinic receptor subtypes yielded a significant and comparable correlation for M_1, M_3, M_4 and M_5 receptors. These results suggest that the postsynaptic receptors in circular muscle of the pig gastric fundus belong to the M_3 subtype. However, the presynaptic receptor could not be clearly defined, although it does certainly not belong to the M_2 subtype.

4.2. Introduction

In circular muscle strips of the pig gastric fundus, contractions induced by electrical field stimulation (EFS) are blocked by atropine (Leclere & Lefebvre, 1998), while the

electrically-evoked release of acetylcholine in the same tissue is enhanced by atropine (Leclere & Lefebvre, 2001), indicating the presence of respectively postsynaptic muscular muscarinic receptors and presynaptic inhibitory muscarinic auto-receptors on the cholinergic neurones of the pig gastric fundus. However, the type of muscarinic receptor(s) involved in the pig gastric fundus has not yet been characterised. Five different muscarinic receptor subtypes have been identified based on studies of molecular structure, *in vitro* binding and function (Buckley *et al.*, 1989; Dörje *et al.*, 1991). All subtypes belong to the seven-transmembrane G-protein coupled receptor family (see reviews Grimm *et al.*, 1994; Eglen *et al.*, 1996; Caulfield & Birdsall, 1998). In most tissues, including the gastrointestinal tract, mainly M_3 receptors can be detected to function pharmacologically at the postsynaptic level (Eglen *et al.*, 1996; Caulfield & Birdsall, 1998). With regard to the presynaptic level, the situation is more complex as both stimulatory and inhibitory muscarinic auto-receptors can be present, and only a limited number of experiments have been reported in the gastrointestinal tract. In guinea-pig longitudinal muscle myenteric plexus (LMMP) preparations, it has been shown that presynaptic muscarinic M_3 receptors inhibit while muscarinic M_1 receptors enhance acetylcholine release (Soejima *et al.*, 1993). However, in circular smooth muscle of the guinea-pig ileum, the presynaptic inhibitory muscarinic receptors belong to the M_1 subtype (Dietrich & Kilbinger, 1995). In the guinea-pig stomach, there is evidence that acetylcholine release is inhibited by muscarinic M_1 and M_2 receptors (Ogishima *et al.*, 2000). In canine LMMP preparations, a binding study demonstrated that presynaptic muscarinic receptors belong to the M_3 subtype, although this technique could not exclude the presence of another presynaptic subtype (Kostka *et al.*, 1989). Prejunctional stimulatory and inhibitory muscarinic receptors have also been demonstrated in other tissues, especially in the respiratory tract and urinary bladder (see reviews Grimm *et al.*, 1994; Somogyi & de Groat, 1999).

By use of functional and release experiments, the present study had two objectives: First, to characterise the muscular muscarinic receptor, responsible for the contraction by muscarinic agonists of the smooth muscle cells in the pig gastric fundus. Second, to characterise the muscarinic receptor(s) present on the cholinergic nerves, inhibiting the release of acetylcholine.

4.3. Methods

4.3.1. Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the pig gastric fundus. The stomach was removed from healthy castrated male pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution (PSS). After the mucosa was removed, strips of approximately 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular muscle except in one series of experiments when strips were cut in the direction of the longitudinal muscle. In one series, circular muscle strips were prepared after removal of the longitudinal muscle layer and the myenteric plexus. All strips were used within 24 hours. When tissues were used the next day, they were stored in fresh PSS at 4°C. Strips were mounted vertically between two platinum plate electrodes (30 x 6 x 0.1 mm) in 20 ml organ baths (functional experiments) or between two platinum wire electrodes (45 x 0.5 mm) in 2 ml organ baths (functional or release experiments) under a load of 2 g, containing PSS (mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$), maintained at 37°C and gassed with carbogen. The PSS contained guanethidine ($4x10^{-6}$ M) to avoid noradrenergic influences, L-N^G-nitroarginine methyl ester (L-NAME, $3x10^{-4}$ M) to prevent relaxations due to nitric oxide, and, as the basal tonus increases during the course of the experiment (Leclere & Lefebvre, 1998), indomethacin (10^{-5} M) to prevent this increase. The mechanical activity of the preparations was recorded *via* isotonic transducers (T_3, Palmer Bioscience, U.S.A.) on a recorder (FWR 3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Japan or Ankersmit, The Netherlands). Electrical field stimulation (EFS) was applied by means of a S88 stimulator (Grass, U.S.A.).

4.3.2. Functional experiments

The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment. After the equilibration period, strips were first maximally contracted with 80 mM KCl, followed by rinsing every 10 min during 30 min.

In a first set of experiments, muscarinic antagonists were tested *versus* electrically and acetylcholine-induced contractions. After equilibration and KCl treatment, EFS (40 V, 0.25

ms, 4 Hz during 2 min) was applied, contracting the tissues. Three to five consecutive electrical stimulations at 10 min intervals were required before contractions remained stable. When the response of muscarinic antagonists *versus* exogenous acetylcholine was studied, a cumulative concentration-response curve for acetylcholine was performed after the electrical stimulations, to determine the concentration of acetylcholine inducing a contraction of similar amplitude as that induced by EFS ($3x10^{-7}$ to 10^{-5} M). Tissues were then re-exposed to this concentration of acetylcholine. After contraction to EFS or exogenous acetylcholine, tissues were washed to re-establish the basal tone level. To study the influence of tetrodotoxin (TTX; $3x10^{-6}$ M), hexamethonium ($5x10^{-4}$ M) and atropine (10^{-6} M) on the electrically-evoked contractions and on the contractions by exogenous acetylcholine, they were added for 30 min before tissues were stimulated again with either EFS or exogenous acetylcholine. To study the influence of muscarinic antagonists, EFS or addition of acetylcholine was repeated at 45 min intervals and increasing concentrations of muscarinic antagonists or solvent were added 30 min before EFS or acetylcholine addition. The response before the first addition of antagonist was used as control response (S_1).

In a second set of experiments, muscarinic antagonists were tested *versus* cumulative concentration-response curves of acetylcholine in order to determine pA_2 values for the postsynaptic muscarinic receptors. After the equilibration period and KCl treatment, tissues were exposed to 10^{-6} M of acetylcholine to test their viability and responsiveness. After washing until basal tone level was re-established, cumulative concentration-response curves to acetylcholine ($3x10^{-8}$ – 10^{-2} M) were constructed using half-logarithmic dosing increments of acetylcholine. After construction of the first concentration-response curve, the preparation was washed for 45 min, until the tension returned to baseline. A muscarinic antagonist was then incubated for 30 min before the second concentration-response curve was obtained. This cycle was repeated four times with increasing concentrations of antagonist. Parallel control experiments without antagonist were performed under identical conditions. In another series of experiments, cumulative concentration-response curves to acetylcholine ($3x10^{-8}$ – 10^{-3} M) or KCl ($5x10^{-3}$ – $8x10^{-2}$ M) were constructed. After construction of the first concentration-response curve, the preparation was washed for 45 min, until the tension returned to baseline. The M_4-selective antagonist MT-3 was then incubated for 30 min before the second concentration-response curve to acetylcholine or KCl was obtained. Parallel control experiments without antagonist were performed under identical conditions. The control experiments allowed for any correction to be made for changes in sensitivity to acetylcholine or KCl.

4.3.3. Release experiments

Strips, weighing 148 ± 5 mg (n = 82), were mounted vertically between two platinum wire electrodes in 2 ml organ baths containing PSS. The PSS contained in addition to the composition mentioned under Tissue preparation $1.5x10^{-6}$ M choline and $5.7x10^{-5}$ M ascorbic acid. Baths were maintained at 37 °C and gassed with carbogen. The tissues were superfused at a rate of 2 ml/min using a peristaltic pump (Gilson Minipuls, France) during 60 min. The strips were subjected to continuous EFS (40 V, 1 ms, 0.5 Hz) during the last 20 min. After the equilibration period, superfusion was stopped and the preparations were incubated for 30 min with [^{3}H]-choline (5 µCi/ml) during which the tissues were stimulated electrically (40 V, 1 ms, 2 Hz) in order to label their cholinergic transmitter stores.

After the labelling procedure, the strips were superfused (2 ml/min) for 90 min with PSS to remove loosely bound radioactivity. From now on the PSS contained in addition 10^{-5} M hemicholinium-3 to prevent the re-uptake of choline. After the washout period, strips were no longer superfused but the content of the organ bath, filled with 1 ml, was collected and replaced each 3 min. A total of 75 samples was collected. 0.5 ml of the samples was mixed with 2 ml of the scintillator containing solution Ultima Gold (Canberra Packard, U.S.A.). The strips were stimulated five times for 2 min, except in one series of experiments with MT-3 when strips were only stimulated four times (S_1 - S_5; 40 V, 0.25 ms, 4 Hz), at 10 min (S_1, 4th sample), 58 min (S_2, 20th sample), 106 min (S_3, 36th sample), 154 min (S_4, 52nd sample), and 202 min (S_5, 68th sample) after the end of the washout period. Muscarinic antagonists were added in increasing concentrations 30 min before S_2 - S_5, and they remained present until the next concentration was added, or until the end of the experiment. At the end of the experiment, tissues were blotted and weighed.

Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Canberra Packard, U.S.A.), and external standardization was used to correct for counting efficiency. Electrical stimulation induced an increase in tritium overflow, not only during stimulation, but also in three samples following that with stimulation. The stimulation-induced increase in tritium overflow was calculated from the difference between the total tritium release during stimulation plus the following three samples, and the calculated basal tritium overflow. Basal tritium overflow during the period of enhanced tritium overflow was calculated by fitting a regression line through the values of the three samples just before stimulation and the values of the three samples starting from where basal release was re-established.

4.3.4. Data analysis

Experimental data are expressed as means ± s.e.mean and *n* refers to the number of the tissues from different animals. For both functional and release experiments, concentration-response curves for the muscarinic antagonists were constructed by expressing the ratio S_n/S_1 in the presence of an antagonist as a percentage of the equivalent ratio obtained in parallel tissues in the absence of antagonists. The concentrations which produced half-maximal inhibition of contractions or facilitation of tritium release (IC_{50}) were calculated by linear interpolation from individual concentration-response curves. Statistical significance ($P < 0.05$) was assessed by the paired and unpaired *t*-test.

In experiments to determine the pA_2 values, the second till fifth concentration-response curves to acetylcholine were expressed as percentage of the maximal contraction in the first concentration-response curve. The EC_{50} was calculated for each curve and the dose ratios (DR) were calculated as $(EC_{50})_n/(EC_{50})_1$. As a moderate rightward shift of the concentration-response curves to acetylcholine occurred in control tissues, the DR in the presence of antagonist was corrected for this change by dividing the DR for tissues in the presence of antagonists by the DR obtained in parallel control tissues. Finally, the log (DR-1) was expressed as a function of log [antagonist], and the pA_2 value was calculated according to Arunlakshana & Schild (1959). The slope of the Schild plot was considered to be not different from unity when the 95% confidence interval for the slope includes 1.0. Then, pK_B values were obtained from plots constrained to a slope of 1.0.

Pearson correlation coefficients (r) and associated *P*-values were calculated using the program GraphPad Prism, version 3.0, for the relationship of binding affinity data generated at the five human recombinant muscarinic receptors with our potency and affinity data.

4.3.5. Drugs used

Acetylcholine chloride, atropine sulphate, choline chloride, dimethylsulfoxide (DMSO), guanethidine sulphate, indomethacin and L-N^G-nitroarginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO, U.S.A.), hemicholinium-3-bromide, p-fluoro-hexahydro-sila-difenidol hydrochloride (p-F-HHSiD), methoctramine 4 hydrochloride and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) were obtained from RBI (Natick, U.S.A.), (11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3b] [1,4]-benzodiazepine-6-one) (AF-DX 116) was obtained from Karl Thomae

GmbH (Biberach, Germany), pirenzepine dihydrochloride from Boehringer Ingelheim (Brussels, Belgium), [methyl-^{3}H]-choline chloride (2775 GBq/mmol) from NEN (Boston, U.S.A.), hexamethonium chloride from Federa (Brussels, Belgium) and mamba toxin-3 (MT-3) and tetrodotoxin (TTX) from Alomone labs (Jerusalem, Israel).

Drugs were dissolved and diluted with distilled water, except for 4-DAMP and AF-DX 116 which were dissolved in DMSO before dilution with distilled water. Stock solutions of 10^{-3} M TTX, 10^{-2} M of atropine, pirenzepine, methoctramine and p-F-HHSiD were kept frozen at –20°C and 10^{-6} M MT-3 was kept at 4°C. Dilutions were made the day of the experiment.

4.4. Results

4.4.1. General observations

Tissues responded to EFS (40 V, 0.25 ms, 4 Hz, 2 min) in the presence of L-NAME ($3x10^{-4}$ M) with a biphasic contraction. Fast contractions occurred in all tissues stimulated, but the initial contraction was sometimes followed by a small decrease of tone and a more sustained contraction whereof the amplitude was smaller than that of the initial phasic contraction; in other tissues the initial contraction was very shortly stopped before the contraction gradually increased to an amplitude higher than the initial contraction. When ending the electrical stimulation, tone declined as quickly as it had risen. The highest amplitude of the electrically-induced contractions was measured. After 3 to 5 electrical stimulations, the contraction amplitude was stable. Addition of exogenous acetylcholine (range $3x10^{-7}$ M till 10^{-5} M) caused monophasic reproducible contractions.

In control tissues of the release experiments, the release of tritium before S_1 (sample 3) was 660 ± 50 Bq per g tissue (n = 14). The amount of tritium released due to S_1 (samples 5, 6, 7 and 8) above calculated basal release (2 590 ± 180 Bq per g tissue) was 2 700 ± 520 Bq per g tissue (n = 14). The outflow evoked by S_2, S_3, S_4 and S_5 was 0.80 ± 0.02, 0.65 ± 0.02, 0.54 ± 0.02 and 0.43 ± 0.02, respectively (n = 14) of that caused by S_1. In the experiments with AF-DX 116 and 4-DAMP, where parallel control tissues received dilutions of the solvent DMSO, these values were not significantly different from values of control tissues receiving aqua as solvent.

4.4.2. Effect of muscarinic receptor antagonists on acetylcholine-induced contractions and on electrically-induced tritium outflow and contractions

Contractions induced by a fixed concentration of acetylcholine were not influenced by TTX ($3x10^{-6}$ M) and hexamethonium ($5x10^{-4}$ M), while atropine (10^{-6} M) completely blocked the contractions, indicating that acetylcholine causes contraction by stimulation of postsynaptic muscular muscarinic receptors, and thus the effect on the contractions by muscarinic antagonists is solely at the postsynaptic level. As shown in Figure 4.1A and 4.2, atropine, pirenzepine, AF-DX 116, 4-DAMP and p-F-HHSiD inhibited the acetylcholine-induced contractions at all concentrations tested, while methoctramine inhibited the acetylcholine-induced contractions only significantly from 10^{-5} M upwards ($n = 6$; $P < 0.05$; Figure 4.2C). MT-3 ($3x10^{-11}$ – 10^{-8} M) did not influence the acetylcholine-induced contractions ($n = 6$; data not shown). The negative logarithms of the postsynaptic IC_{50} values of the antagonists, determined *versus* acetylcholine, are given in Table 4.1. The rank order of potency of the antagonists at postsynaptic level was: atropine = 4-DAMP > p-F-HHSiD > pirenzepine > AF-DX 116 > methoctramine.

In release studies, the electrically-evoked tritium outflow was abolished by TTX, ω-conotoxin-GVIA or removal of extracellular calcium, and contains a consistent amount of acetylcholine as determined by HPLC (Leclere & Lefebvre, 2001), indicating that electrical stimulation activates cholinergic neurones releasing acetylcholine. This acetylcholine inhibits its own release by activation of presynaptic muscarinic receptors on the cholinergic neurones (Leclere & Lefebvre, 2001), and changes in electrically-induced tritium release by muscarinic antagonists are only related to interference with these presynaptic muscarinic receptors on these cholinergic neurones. None of the muscarinic antagonists affected the basal outflow of tritium, while all antagonists, except MT-3, increased concentration-dependently the electrically-evoked tritium outflow (Figure 4.2). Methoctramine and p-F-HHSiD showed bell-shaped concentration-response curves. MT-3 ($3x10^{-11}$ – 10^{-8} M) did not influence the electrically-induced tritium release ($n = 6$; data not shown). However, higher concentrations of MT-3 decreased the electrically-induced release (10^{-8} M: 101 ± 2%; $3x10^{-8}$ M: 87 ± 2% ($P < 0.01$); 10^{-7} M: 81 ± 3% ($P < 0.01$) ($n = 6$)). The negative logarithms of the presynaptic IC_{50} values of the antagonists are given in Table 4.1. The rank order of potency of the antagonists at presynaptic level was: 4-DAMP > atropine > pirenzepine ≥ p-F-HHSiD > AF-DX 116 > methoctramine. As acetylcholine release from cholinergic nerve endings towards the longitudinal muscle layer might contribute to the tritium measured in the whole tissue strips, a

small series of experiments measuring tritium release was performed in the absence of the longitudinal muscle layer and myenteric plexus so that only the tritium release of nerve endings in the circular muscle layer was evaluated (n = 4; data not shown). The amount of tritium released was too low for obtaining reproducible results.

The electrically-induced contractions were completely blocked by atropine (10^{-6} M) and TTX ($3x10^{-6}$ M), while hexamethonium ($5x10^{-4}$ M) was without effect, indicating the activation of postganglionic cholinergic neurones during EFS. The released acetylcholine

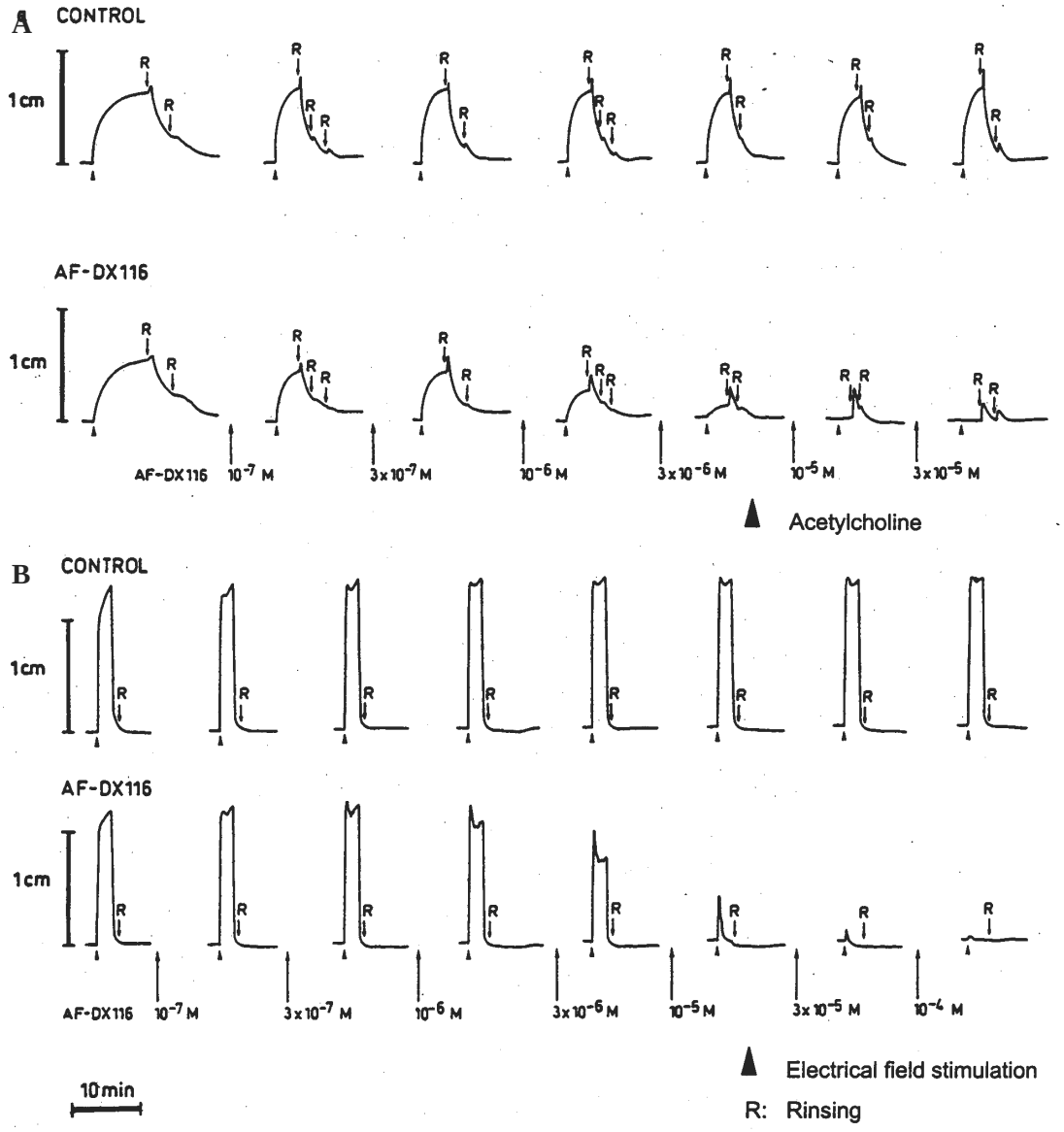

Figure 4.1 Representative traces showing the contractions to exogenous acetylcholine ($3x10^{-7}$ M) (A) and electrical field stimulation (B) in the presence of solvent or increasing concentrations of AF-DX 116. AF-DX 116 was incubated for 30 min before tissues were stimulated.

causes contraction by stimulating postsynaptic muscular muscarinic receptors but this contraction will be influenced by the effect of the released acetylcholine on its own release by stimulation of presynaptic muscarinic receptors on the cholinergic neurones. Antagonists tested *versus* the electrically-evoked contractions can thus interfere with the response at both pre- and postsynaptic muscarinic receptors. The results are shown in Figure 4.2 and the pIC_{50} values are given in Table 4.1. Pirenzepine and 4-DAMP concentration-dependently inhibited the contractions induced by EFS and the concentration-response curve paralleled that *versus* the acetylcholine-induced contractions. In contrast, lower concentrations of methoctramine and AF-DX 116 (Figure 4.1B) increased the amplitude of the electrically-induced contractions, while higher concentrations decreased it. Although atropine and p-F-HHSiD did not enhance the electrically-induced contractions, the concentration-response curve *versus* these contractions was moderately (atropine) to markedly (p-F-HHSiD) shifted to the right in comparison with that *versus* acetylcholine-induced contractions. MT-3 ($3x10^{-11}$ – 10^{-8} M) did not influence the electrically-induced contractions (n = 6; data not shown). However, higher concentrations tended to increase the electrically-induced contractions (10^{-8} M: 100 ± 4%; $3x10^{-8}$ M: 101 ± 6%; 10^{-7} M: 108 ± 7% (n = 4)).

The effect of pirenzepine was also studied on electrically- and acetylcholine-induced contractions in strips cut in the longitudinal direction so that the contractions were due to longitudinal smooth muscle activity. In these tissues, the pIC_{50} value of pirenzepine *versus* electrically-induced contractions (6.55 ± 0.13; n = 4) was significantly larger than that *versus* acetylcholine-induced contractions (6.06 ± 0.12; n = 4; $P < 0.05$).

Table 4.1 Comparison between pre- and postsynaptic potencies (pIC_{50}) of muscarinic antagonists in circular muscle strips of the pig gastric fundus

Antagonist	[1]$pIC_{50\ pre}$	n	[2]$pIC_{50\ post}$	n	[3]$pIC_{50\ (EFS)}$	n
Atropine	8.12 ± 0.18	6	8.48 ± 0.11	8	$8.10 \pm 0.12^{+}$	8
Pirenzepine	7.09 ± 0.05	6	6.37 ± 0.12***	8	6.35 ± 0.06***	8
AF-DX116	6.65 ± 0.32	6	5.69 ± 0.08**	8	$5.03 \pm 0.08^{***,+++}$	8
Methoctramine	6.43 ± 0.14	7	5.19 ± 0.14***	6	$4.72 \pm 0.07^{***,++}$	8
4-DAMP	8.25 ± 0.04	6	8.48 ± 0.08*	8	8.54 ± 0.07**	8
p-F-HHSiD	7.03 ± 0.30	6	6.85 ± 0.13	8	$6.09 \pm 0.11^{**,+++}$	7

The pIC_{50} values are mean ± s.e.mean. 1: Assessed versus EFS-induced tritium release; 2: Assessed versus acetylcholine-induced contraction; and 3: Assessed versus EFS-induced contraction. *,**,***: $P < 0.05$, 0.01, 0.001. Significantly different from $pIC_{50\ pre}$. +,++,+++: $P < 0.05$, 0.01, 0.001. Significantly different from $pIC_{50\ post}$.

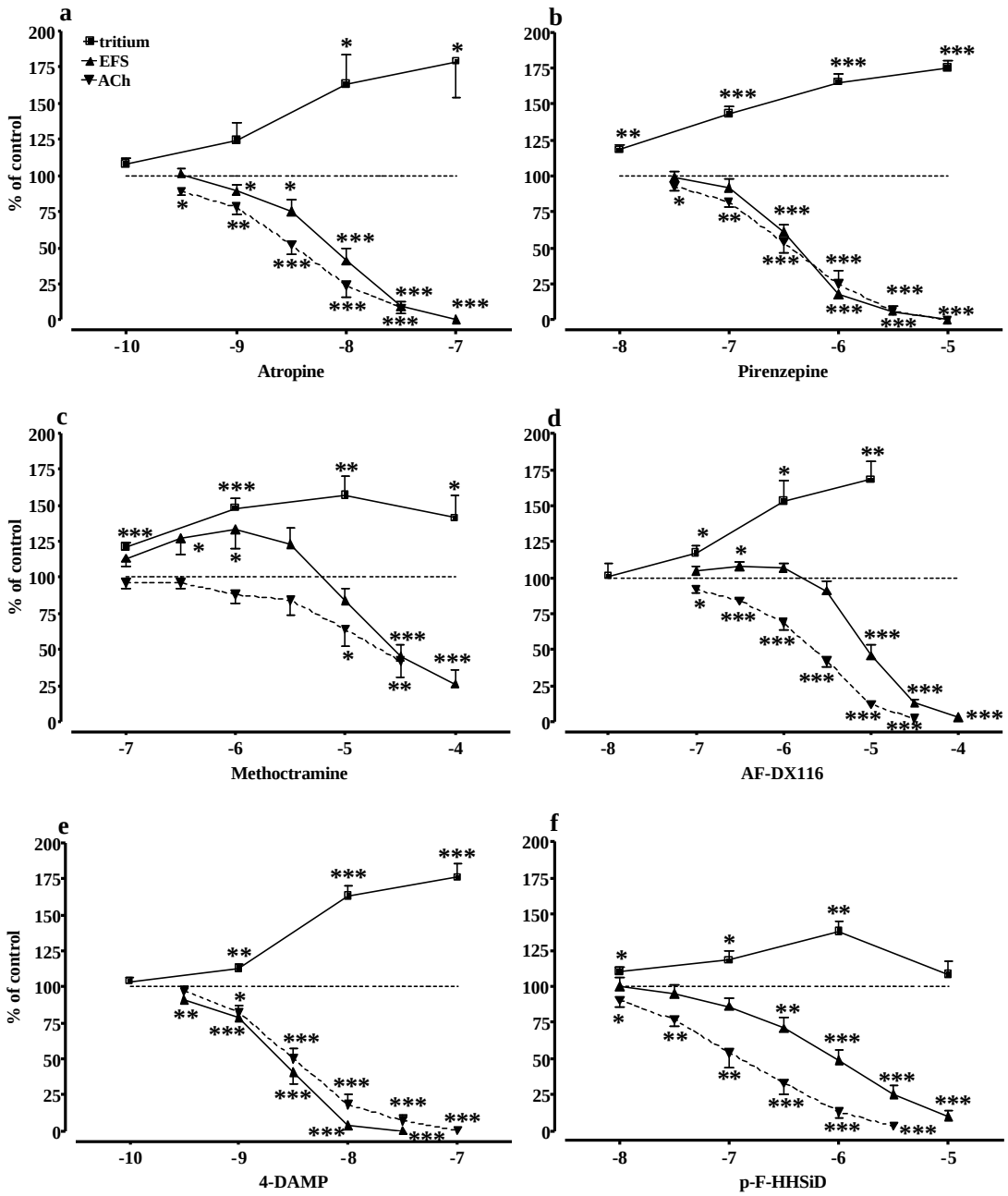

Figure 4.2 Mean ± s.e.mean (n = 6 to 8) of the effect of muscarinic antagonists on tritium outflow (tritium) induced by electrical stimulation and smooth muscle contraction evoked by either electrical field stimulation (EFS) (40 V, 0.25 ms, 4 Hz, 2 min) or exogenous acetylcholine (ACh) in the circular muscle of pig gastric fundus strips. The ratio of the response in the presence of antagonist versus the control response before addition of antagonist (S_n/S_1) was expressed as % of the same ratio in parallel control tissues receiving the solvent of the antagonist. *,**,***: P < 0.05, 0.01, 0.001: Significantly different from the value in the absence of antagonist (= 100 %).

4.4.3. Postsynaptic affinities of muscarinic receptor antagonists versus acetylcholine

Acetylcholine caused concentration-dependent contractions with an EC_{50} of 2.05 ± 0.41x10^{-6} M (n = 40), and the maximal contraction was 111 ± 1% as percentage of the KCl

(80 mM)-induced contraction (n = 40). Except for MT-3 ($3x10^{-10}$ – 10^{-8} M; n = 2), parallel rightward shifts of the concentration-response curves to acetylcholine, without depression of the maximum response, were obtained with the muscarinic antagonists (atropine: $3x10^{-9}$ – 10^{-7} M, n = 6; pirenzepine: 10^{-7} – $3x10^{-6}$ M, n = 6; AF-DX 116: 10^{-6} – $3x10^{-5}$ M, n = 6; methoctramine: $3x10^{-6}$ – $3x10^{-5}$ M, n = 8; 4-DAMP: 10^{-9} – $3x10^{-8}$ M, n = 8 (Figure 4.3A); p-F-HHSiD : 10^{-7} – $3x10^{-6}$ M, n = 8 (Figure 4.3B)). In two out of eight tissues, methoctramine ($3x10^{-6}$ – $3x10^{-5}$ M) produced no significant parallel rightward shift of the concentration-response curve. The Schild plot was therefore performed on the basis of the results in the six tissues where methoctramine was active. Schild regression analysis was linear with a slope not significantly different from unity for atropine, pirenzepine, 4-DAMP and p-F-HHSiD (Table 4.2). The rank order of antagonist affinities was 4-DAMP > atropine > p-F-HHSiD > pirenzepine > AF-DX 116 > methoctramine.

MT-3 (10^{-7} M; n = 4) caused a parallel leftward shift of the concentration-response curve to acetylcholine, without depression of the maximum response. The pEC_{50} of acetylcholine in the control strips was 5.54 ± 0.19, while this was 6.25 ± 0.18 in the presence of MT-3 ($P < 0.05$). However, MT-3 (10^{-7}; n = 4) had no effect on the concentration-response curve to KCl. The pEC_{50} of the control strips was 1.67 ± 0.04, while this was 1.75 ± 0.02 in the presence of MT-3.

4.5. Discussion

In this study we describe the pharmacological profile of muscarinic receptor(s) involved in the control of acetylcholine release and smooth muscle contraction of the circular

Table 4.2 Postsynaptic affinity values of muscarinic antagonists at receptors mediating contraction of circular muscle strips of the pig gastric fundus in response to acetylcholine

Antagonist	pA_2	Slope	pK_B	n
Atropine	8.94 ± 0.07	0.87 ± 0.07	8.79 ± 0.04	6
Pirenzepine	6.76 ± 0.19	0.98 ± 0.15	6.70 ± 0.09	6
AF-DX116	5.96 ± 0.12	0.71 ± 0.07		6
Methoctramine	5.55 ± 0.25	0.20 ± 0.09		6
4-DAMP	9.09 ± 0.16	1.05 ± 0.09	9.13 ± 0.05	8
p-F-HHSiD	7.13 ± 0.13	0.94 ± 0.12	7.01 ± 0.08	8

Values are means ± s.e.mean. The antagonist action of each antagonist was estimated by Schild plot analysis, and pA_2 values and the slope of the linear regression were calculated. When the 95 % confidence limits of the slope included the value 1.0, the line slope was considered to be not significantly different from unity, and pK_B values were obtained from plots constrained to a slope of 1.0.

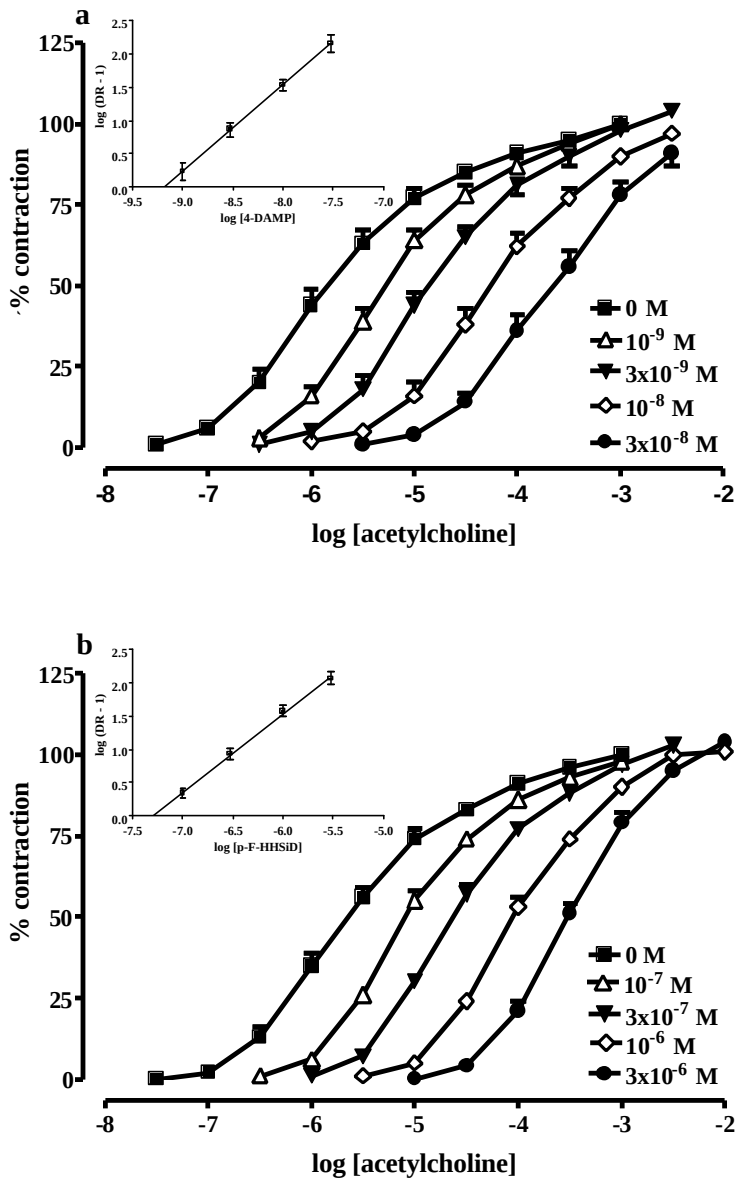

Figure 4.3 Cumulative concentration-response curves for acetylcholine-induced contractions, expressed as a percentage of the maximum to acetylcholine in the first concentration-response curve, in the circular muscle of pig gastric fundus strips in the absence and presence of different concentrations of 4-DAMP (A) and p-F-HHSiD (B). Inserts show the Schild plots of the respective antagonists. The pA_2 values and slopes are given in Table 4.2. Data points represent means ± s.e.mean of 8 independent experiments.

muscle of the pig gastric fundus. Because of the lack of selective muscarinic antagonists, a series of subtype-preferring antagonists are used to characterise pharmacologically the involvement of a particular muscarinic receptor. The muscarinic antagonists used in this study to determine the profile of the pre- and postsynaptic muscarinic receptors of the circular muscle of the pig gastric fundus are the non-selective antagonist atropine, the M_1-preferring

antagonist pirenzepine, the M_2-preferring antagonists AF-DX 116 and methoctramine, the M_3-preferring antagonists 4-DAMP and p-F-HHSiD and the M_4-preferring antagonist MT-3.

As described in the Results section, the postsynaptic muscarinic receptors are assessed when studying the influence of the muscarinic antagonists on acetylcholine-induced contractions. The tissues are mounted in the direction of the circular muscle layer to measure these contractions and the short parts of longitudinal muscle in the tissues will not contribute to the contractions; this series of experiments thus evaluates the postsynaptic muscarinic receptors on the circular muscle cells of the pig gastric fundus. The presynaptic muscarinic receptors are assessed when studying the influence of the muscarinic antagonists on electrically-induced tritium release; cholinergic neurones directed to the longitudinal muscle might contribute to the tritium release in this series, and interference of the muscarinic antagonists with presynaptic muscarinic receptors on these neurones might influence the results. The release experiments were performed in the absence of the acetylcholinesterase inhibitor physostigmine. We indeed previously demonstrated (Leclere & Lefebvre, 2001) that the S_n/S_1 ratio for tritium is systematically similar to that for [^{3}H]-acetylcholine, so it is not necessary to separate the radioactive components, which requires the presence of physostigmine. Muscarinic antagonists can interfere with both pre- and postsynaptic muscarinic receptors when studied *versus* electrically-induced contractions, as illustrated by the results with atropine. The pIC_{50} of atropine *versus* electrically-induced contractions was significantly lower than the pIC_{50} *versus* acetylcholine-induced contractions; the stimulatory effect on acetylcholine release by antagonism of the presynaptic muscarinic receptors will indeed counteract the antagonistic effect *versus* acetylcholine at the postsynaptic level. As the measured item is contraction, the presynaptic muscarinic receptors on the cholinergic neurones to the longitudinal muscle will not interfere in this assay.

4.5.1. Characterisation of the postsynaptic muscarinic receptors

The rank order of potencies (Table 4.1) and affinities (Table 4.2) at the postsynaptic level of the six investigated subtype-preferring antagonists is similar, and comparing these rank orders with the rank order of the binding constants for the five muscarinic receptor subtypes (Table 4.3) is consistent with the pharmacological profile of the M_3 and M_5 receptor subtypes. However, analysis of the relationship of our potency and affinity values with literature data of binding affinity values for the muscarinic receptor subtypes shows that the best correlation coefficient (potency: M_3: r = 0.977, P = 0.001; M_5: r = 0.930, P = 0.007 and

affinity: M_3: r = 0.977, P = 0.001; M_5: r = 0.941, P = 0.005) was found for the M_3 subtype. Our conclusion is similar to findings in gastrointestinal smooth muscle preparations (e.a. Lazareno & Roberts, 1989; Doods *et al.*, 1994; Preiksaitis & Laurier, 1998; Shi & Sarna, 1997, 1999) and in other tissues (see review Eglen *et al.*, 1996), where the postsynaptic muscarinic receptors also belong to the M_3 subtype. The slope of the Schild plot for AF-DX 116 and especially for methoctramine was very small. In the guinea-pig LMMP preparations, the slope for methoctramine *versus* acetylcholine-induced contractions was also significantly less than unity (Barocelli *et al.*, 1994). One possible explanation might be the antagonism of a heterogenous receptor population (Kenakin, 1993). Indeed, many investigators demonstrated the presence of M_2 receptors together with M_3 receptors on smooth muscle *via* molecular and radioligand binding studies, or pharmacologically *via* indirect methods (see reviews Ehlert *et al.*, 1999; Eglen, 2001). Although the majority of the muscular muscarinic receptors belong to the M_2 subtype, muscarinic agonists mainly cause contraction *via* stimulation of the M_3 receptors. Stimulation of M_2 receptors inhibits adenylyl cyclase, and they will oppose the relaxations due to activation of adenylyl cyclase by e.g. stimulation of β-adrenoceptors (Ehlert *et al.*, 1999; Eglen, 2001). The M_2 receptors might also have an important effect during inflammation (Shi & Sarna, 1997, 1999). However, when using the method described by Hegde *et al.* (1997) to determine the presence of these M_2 receptors, no evidence was found for the presence of M_2 receptors on the pig gastric fundus (results not shown).

Table 4.3 Affinity values (pK_i) at muscarinic receptors from literature

	M_1	M_2	M_3	M_4	M_5
Atropine	9.27	8.96	9.39	9.11	9.11
Pirenzepine	7.96	6.24	6.82	7.11	6.73
AF-DX 116	6.44	7.20	6.07	6.68	5.29
Methoctramine	7.08	7.78	6.40	6.89	6.36
4-DAMP	9.03	8.14	9.28	8.49	8.91
p-F-HHSiD	7.30	6.41	7.56	7.21	6.73
MT-3	6.78	< 6.3	6.3	8.33	

Affinity values (pK_i) are the mean values which refer to radioligand binding studies at cortex (M_1), heart (M_2), submandibular and lacrimal gland (M_3) and human cloned muscarinic receptors expressed in Chinese hamster ovary (CHO) cells (data from Lazareno & Roberts, 1989; Lazareno *et al.*, 1990; Pedder *et al.*, 1991; Doods *et al.*, 1993; Esqueda *et al.*, 1996; Hegde *et al.*, 1997). For MT-3, affinity values (pA_2) refer to the effect of MT-3 on acetylcholine stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing the cloned human M_1 – M_4 receptors (Olianas *et al.*, 1999).

4.5.2. Characterisation of the presynaptic muscarinic receptors

The results of the experiments performed to characterise the presynaptic muscarinic receptors do not allow to strongly support one particular subtype.

The rank order of potencies of the muscarinic antagonists at the presynaptic level, as assessed *via* their influence on tritium release (Table 4.1) corresponds with the pharmacological profile of the M_1 subtype (Table 4.3) and the correlation between the presynaptic pIC_{50} values (Table 4.1) with published binding affinity values for the M_1 receptor (Table 4.3) is significant ($r = 0.941$, $P = 0.005$), but other points argue against M_1 receptors. First, the potency of pirenzepine was almost tenfold lower than expected at M_1 receptors (see Table 4.3). Second, pirenzepine did not discriminate between the electrically- and acetylcholine-induced contractions, suggesting that the auto-receptor is not an M_1 receptor. Nevertheless, the pIC_{50} value for pirenzepine obtained *versus* acetylcholine-induced contractions was significantly lower than that on tritium outflow. This might be due to the presence of inhibitory M_1 receptors on cholinergic neurones innervating the longitudinal smooth muscle of the pig gastric fundus and contributing to the higher potency of pirenzepine on tritium outflow. Indeed, a different type of presynaptic muscarinic receptors has already been demonstrated on nerve endings in the circular *versus* longitudinal smooth muscle of the guinea-pig ileum (Soejima *et al.*, 1993; Dietrich & Kilbinger, 1995). Release experiments in circular muscle strips without longitudinal muscle layer and myenteric plexus, to avoid the possible interference from the cholinergic nerve endings in the longitudinal muscle, did not yield reproducible results. Therefore, pirenzepine was tested in strips cut in the direction of the longitudinal muscle layer. The pIC_{50} value of pirenzepine *versus* electrically-induced contractions was larger than those *versus* acetylcholine-induced contractions in these strips, suggesting that facilitatory M_1 receptors are present on the cholinergic neurones innervating the longitudinal muscle. We have thus no explanation for the results with pirenzepine in the circular muscle strips.

When looking at the pIC_{50} values (Table 4.1), the M_2 subtype preferring muscarinic antagonists AF-DX 116 and methoctramine were more potent in facilitating the evoked tritium release than in inhibiting the contractile response. Also, electrically-evoked contractions were enhanced at the lowest concentrations, presumably due to facilitation of acetylcholine release. These results would correlate with the presence of presynaptic M_2 receptors, but this possibility cannot be maintained. Indeed 1) The rank order of the pIC_{50} values *versus* electrically-induced tritium release did not correspond with that of an M_2

receptor; and 2) When comparing the presynaptic pIC_{50} values with published pK_i values for the M_2 muscarinic subtype ($r = 0.581$, $P = 0.227$), no significant correlation was found.

With regard to M_4 receptors, a significant correlation was found between our presynaptic pIC_{50} values and the pK_i values from literature ($r = 0.944$, $P = 0.005$). However, it is not possible to define with certainty that the presynaptic muscarinic receptor belongs to the M_4 subtype. Indeed, the M_4 antagonist MT-3 had no effect on the electrically-induced contractions and tritium release up to 10^{-8} M. As the pK_i value of MT-3 on M_4 receptors is 8.33 (Olianas et al., 1999), some effect of 10^{-8} M can be expected. Indeed, D'agostino et al. (2000) demonstrated that in the human detrusor MT-3 increases [^{3}H]-acetylcholine release by acting at the M_4 receptor with a pIC_{50} value of 8.50, also illustrating that the substance penetrates in muscle strips. Higher concentrations of MT-3 ($3x10^{-8}$ – 10^{-7} M) even decreased the electrically-induced tritium release in our study. This would correlate with the presence of facilitatory M_4 receptors rather than inhibitory M_4 receptors on the cholinergic neurones. However, one might then also expect a decrease in the electrically-induced tritium release at higher concentrations of atropine, pirenzepine, AF-DX 116 and 4-DAMP in view of their capacity at M_4 receptors (see Table 4.3). Another possibility is that MT-3, which is a toxin (Adem & Karlsson, 1997; Jerusalinsky et al., 2000), has a toxic effect at these concentrations. Remarkably, although the electrically-induced release decreased at these high concentrations of MT-3, the electrically-induced contractions tended to increase. This is probably due to a postsynaptic effect of MT-3 whereby MT-3 interferes with the signal transduction of acetylcholine. Indeed, MT-3 caused a leftward shift of the concentration-response curve to acetylcholine, while it did not influence the concentration-response curve to KCl.

With regard to M_3 receptors, the presynaptic pIC_{50} values of the antagonists used correlated closely with the average of the binding affinities for the M_3 receptor ($r = 0.965$, $P = 0.002$), suggesting the presence of presynaptic M_3 receptors, inhibiting the release of acetylcholine. This supports findings in the guinea-pig and canine LMMP preparations, where presynaptic M_3 receptors were responsible for the inhibition of acetylcholine release (Soejima *et al.*, 1993; Kostka *et al.*, 1989). However, it should be noted that a good correlation was also obtained with the average of binding affinity values for the cloned human M_5 receptor ($r = 0.945$, $P = 0.005$). This may not surprise given the fact that most antagonists discriminate poorly between M_3 and M_5 receptors. It can also not be excluded that more than one subtype of muscarinic receptor is involved in the presynaptic inhibitory control of acetylcholine release.

In conclusion, the postsynaptic contractile muscarinic receptors in the circular muscle of the pig gastric fundus seem to belong to the M_3 receptor subtype, while the presynaptic muscarinic receptor cannot be clearly defined.

4.6. References

ADEM, A. & KARLSSON, E. (1997). Muscarinic receptor subtype selective toxins. *Life Sciences*, **60**, 1069-1076

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.*, **14**, 48-58

BAROCELLI, E., BALLABENI, V., CHIAVARINI, M., MOLINA, E., LAVEZZO, A. & IMPICCIATORE, M. (1994). Muscarinic M_1 and M_3 receptor antagonist effects of a new pirenzepine analogue in isolated guinea-pig ileal longitudinal muscle-myenteric plexus. *Eur. J. Pharmacol.*, **254**, 151-157

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469-476

CAULFIELD, M.P. & BIRDSALL, N.J.M. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.*, **50**, 279-290

D'AGOSTINO, G., BOLOGNESI, M.L., LUCCHELLI, A., VICINI, D., BALESTRA, B., SPELTA, V., MELCHIORRE, C. & TONINI, M. (2000). Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M_4 receptor subtype. *Br. J. Pharmacol.*, **129**, 493-500

DIETRICH, C. & KILBINGER, H. (1995). Prejunctional M1 and postjunctional M3 muscarinic receptors in the circular muscle of the guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **351**, 237-243

DOODS, H.N., ENTZEROTH, M., ZIEGLER, H., MAYER, N. & HOLZER, P. (1994). Pharmacological profile of selective muscarinic receptor antagonists on guinea-pig ileal smooth muscle. *Eur. J. Pharmacol.*, **253**, 275-281

DOODS, H.N., WILLIM, K.D., BODDEKE, H.W.G.M. & ENTZEROTH, M. (1993). Characterization of muscarinic receptors in guinea-pig uterus. *Eur. J. Pharmacol.*, **250**, 223-230

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.R. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **256**, 727-733

EGLEN, R.M. (2001). Muscarinic receptors and gastrointestinal tract smooth muscle function. *Life Sciences*, **68**, 2573-2578

EGLEN, R.M., HEGDE, S.S. & WATSON, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.*, **48**, 531-565

EHLERT, F.J., SAWYER, G.W. & ESQUEDA, E.E. (1999). Contractile role of M_2 and M_3 muscarinic receptors in gastrointestinal smooth muscle. *Life Sciences*, **64**, 387-394

ESQUEDA, GERSTIN, E.H., GRIFFIN, M.T. & EHLERT, F.J., (1996). Stimulation of cyclic AMP accumulation and phosphoinositide hydrolysis by M_3 muscarinic receptors in rat peripheral lung. *Biochem. Pharmacol.*, **52**, 643-658

GRIMM, U., MOSER, E., MUTSCHLER, M.E. & LAMBRECHT, G. (1994). Muscarinic receptors: focus on presynaptic mechanisms and recently developed novel agonists and antagonists. *Pharmazie*, **49**, 711-726

HEGDE, S.S., CHOPPIN, A., BONHAUS, D., BRIAUD, S., LOEB, M., MOY, T.M., LOURY, D. & EGLEN, R.M. (1997). Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **120**, 1409-1418

JERUSALINSKY, D., KORNISIUK, E., ALFARO, P., QUILLFELDT, J., FERREIRA, A., RIAL, V.E., DURAN, R. & CERVENANSKY, C. (2000). Muscarinic toxins: novel pharmacological tools for the muscarinic cholinergic system. *Toxicon*, **38**, 747-761

KENAKIN, T. (1993). Competitive antagonism. In *Pharmacologic Analysis of Drug-Receptor Interaction*. Ed. Kenakin, T. pp. 278-322. New York: Raven Press.

KOSTKA, P., KWAN, C.-Y. & DANIEL, E.E. (1989). Presynaptic and postsynaptic muscarinic receptors in dog ileum: binding studies. *Eur. J. Pharmacol.*, **173**, 35-42

LAZARENO, S., BUCKLEY, N.J. & ROBERTS, F.F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. *Mol. Pharmacol.*, **38**, 805-815

LAZARENO, S. & ROBERTS, F.F. (1989). Functional and binding studies with muscarinic M_2-subtype selective antagonists. *Br. J. Pharmacol.*, **98**, 309-317

LECLERE, P.G. & LEFEBVRE, R.A. (1998). Investigation of the interaction between cholinergic and nitrergic neurotransmission in the pig gastric fundus. *Br. J. Pharmacol.*, **125**, 1779-1787

LECLERE, P.G. & LEFEBVRE, R.A. (2001). Influence of nitric oxide donors and of the α_2-agonist UK-14,304 on acetylcholine release in the pig gastric fundus. *Neuropharmacology*, **40**, 270-278

OGISHIMA, M., KAIBARA, M., UEKI, S., KURIMOTO, T. & TANIYAMA, K. (2000). Z-338 facilitates acetylcholine release from enteric neurons due to blockade of muscarinic autoreceptors in guinea pig stomach. *J. Pharmacol. Exp. Ther.*, **294**, 33-37

OLIANAS, M.C., INGIANNI, A., MAULLU, C., ADEM, A., KARLSSON, E. & ONALI, P. (1999). Selectivity profile of muscarinic toxin 3 in functional assays of cloned and native receptors. *J. Pharmacol. Exp. Ther.*, **288**, 164-170

PEDDER, E.K., EVELEIGH, P., POYNER, D., HULME, E.C. & BIRDSALL, N.J.M. (1991). Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. *Brit. J. Pharmacol.*, **103**, 1561-1567

PREIKSAITIS, H.G. & LAURIER, L.G. (1998). Pharmacological and molecular characterization of muscarinic receptors in cat esophageal smooth muscle. *J. Pharmacol. Exp. Ther.*, **285**, 853-861

SHI, X.-Z. & SARNA, S.K. (1997). Inflammatory modulation of muscarinic receptor activation in canine ileal circular muscle cells. *Gastroenterology*, **112**, 864-874

SHI, X.-Z. & SARNA, S.K. (1999). Differential inflammatory modulation of canine ileal longitudinal and circular muscle cells. *Am. J. Physiol.*, **277**, G341-G350

SOEJIMA, O., KATSURAGI, T. & FURUKAWA, T. (1993). Opposite modulation by muscarinic M_1 and M_3 receptors of acetylcholine release from guinea pig ileum as measured directly. *Eur. J. Pharmacol.*, **249**, 1-6

SOMOGYI, G.T. & DE GROAT, W.C. (1999). Function, signal transduction mechanisms and plasticity of presynaptic muscarinic receptors in the urinary bladder. *Life Sciences*, **64**, 411-418

CHAPTER 5

PRESYNAPTIC MODULATION OF CHOLINERGIC NEUROTRANSMISSION IN THE HUMAN PROXIMAL STOMACH

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CHAPTER 5

PRESYNAPTIC MODULATION OF CHOLINERGIC NEUROTRANSMISSION IN THE HUMAN PROXIMAL STOMACH

5.1. Summary

This study investigates whether the cholinergic neurones, innervating the human proximal stomach, can be modulated by nitric oxide (NO) or vasoactive intestinal polypeptide (VIP), or *via* presynaptic muscarinic, α_2- or 5-hydroxytryptamine$_4$ (5-HT$_4$-) receptors. Circular muscle strips, without mucosa, were incubated with [^{3}H]-choline to incorporate [^{3}H]-acetylcholine into the cholinergic transmitter stores. The basal and electrically-induced release of tritium and [^{3}H]-acetylcholine were analysed in a medium containing guanethidine ($4x10^{-6}$ M), hemicholinium-3 (10^{-5} M), physostigmine (10^{-5} M) and atropine (10^{-6} M). Tissues were stimulated twice for 2 min (S_1 and S_2: 40 V, 1 ms, 4 Hz) and drugs were added before S_2. The NO synthase inhibitor L-N^G-nitroarginine methyl ester ($3x10^{-4}$ M) and the NO donor sodium nitroprusside (10^{-5} M), as well as VIP (10^{-7} M) did not influence the basal release nor the electrically-evoked release. The α_2-adrenoceptor agonist UK-14,304 (10^{-5} M) significantly inhibited the electrically-evoked release of [^{3}H]-acetylcholine, and this was prevented by the α_2-adrenoceptor antagonist rauwolscine ($2x10^{-6}$ M). The 5-HT$_4$-receptor agonist prucalopride ($3x10^{-7}$ M) significantly enhanced the electrically-evoked release of [^{3}H]-acetylcholine, and the 5-HT$_4$-receptor antagonist SB204070 (10^{-9} M) prevented this. When atropine (10^{-6} M) was omitted from the medium and added before the second stimulation, it significantly increased the release of [^{3}H]-acetylcholine. These results suggest that the release of acetylcholine from the cholinergic neurones, innervating the circular muscle in the human proximal stomach, can be inhibited *via* presynaptic muscarinic auto-receptors and α_2-adrenoceptors, and stimulated *via* presynaptic 5-HT$_4$-receptors. No evidence for modulation by NO or VIP was obtained.

5.2. Introduction

With regard to motility, the stomach can be divided into a proximal and a distal part. The proximal stomach consists of the fundus and the orad third of the corpus (Kelly, 1980). It acts as a reservoir for solid and liquid food, and plays a major role in the gastric emptying of

liquids (see review Kelly, 1980). During a meal, the proximal stomach relaxes with minimal increases in intragastric pressure, as inhibitory non-adrenergic non-cholinergic (NANC) neurones become activated (Abrahamsson, 1986). Then, a tonic contraction of the proximal stomach generates a gastroduodenal pressure gradient that has been shown to play an important role in liquid emptying from the stomach (Kelly, 1980; Valenzuela & Liu, 1982). In man, *in vivo* experiments demonstrated that atropine reduces proximal gastric emptying, while the muscarinic agonist bethanechol tended to stimulate proximal stomach contractility (Parkman *et al.*, 1999), suggesting that proximal gastric tone in man appears to be maintained, at least in part, by cholinergic input. In contrast, the proximal gastric relaxation during a meal, as mentioned above, depends on NANC neurones, of which the nitrergic neurones, releasing nitric oxide (NO), are the most important (Lefebvre, 1993; Tonini *et al.*, 2000). Interaction between the nitrergic and cholinergic system might occur at the level of the stomach, as indirect evidence from functional experiments suggests that NO might inhibit the release of acetylcholine from intrinsic cholinergic nerve endings in rat, canine and rabbit gastric fundus (Lefebvre *et al.*, 1992; Baccari *et al.*, 1993; Paterson *et al.*, 2000). In experimental animals, but not in humans, it has been shown that the release of acetylcholine at the level of the stomach can be modulated by different types of presynaptic receptors. Stimulation of presynaptic α_2-adrenoceptors (Jansson & Lisander, 1969; Lefebvre *et al.*, 1984; MacDonald *et al.*, 1990; Leclere & Lefebvre, 2001) and vasoactive intestinal polypeptide (VIP)-receptors (Milenov *et al.*, 1991; Baccari *et al.*, 1994) inhibits the release of acetylcholine, while activation of 5-hydroxytryptamine$_4$ (5-HT$_4$)-receptors increases its release (Amemiya *et al.*, 1996; Matsuyama *et al.*, 1996; Takada *et al.*, 1999). The possibility of auto-inhibition of acetylcholine release by stimulation of presynaptic muscarinic receptors has been demonstrated in the pig and guinea-pig stomach (Ogishima *et al.*, 2000; Leclere & Lefebvre, 2001).

The aim of this study in the human proximal stomach was to measure acetylcholine release directly and to investigate possible presynaptic modulation of acetylcholine release by NO, and *via* presynaptic α_2-adrenoceptors and muscarinic, VIP - and 5-HT$_4$-receptors.

5.3. Methods

5.3.1. Tissue preparation

With the approval of the local ethics committee, macroscopically normal segments of gastric fundus (n = 18) or corpus (n = 2) were obtained from 20 patients (16 men, mean age 63 years (range 30 - 95)) undergoing surgery for oesophageal or gastric carcinoma. Experiments were carried out on isolated circular smooth muscle strips of the human gastric fundus or corpus. As no differences between fundus and corpus were observed, the results are pooled. As soon as possible, the stomach was incubated in physiological salt solution (PSS) and transported to the laboratory. After the mucosa was removed, full thickness strips of 1 to 1.5 cm in length and 0.3 cm in width (weight: 65 ± 3 mg; n = 99) were cut in the direction of the circular muscle. All strips were used within 24 hours, except in one case when strips were used up to 36 hours after surgery. Strips were mounted vertically between two platinum wire electrodes (40 x 0.5 mm) under a load of 2 g in 2 ml organ baths containing PSS (composition in mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose, 25 $NaHCO_3$, 0.0015 choline and 0.057 ascorbic acid), maintained at 37°C and gassed with carbogen (95% O_2 / 5% CO_2). Guanethidine ($4x10^{-6}$ M) was present in the medium throughout all experiments. Electrical field stimulation (EFS) was applied by means of a Grass stimulator (S88, USA).

5.3.2. Experimental protocol

Basically, the same method was used as described for the labelling of acetylcholine pools in pig gastric fundus (Leclere & Lefebvre, 2001). Briefly, during 60 min, the tissues were superfused at a rate of 2 ml/min, using a peristaltic pump (Gilson Minipuls, France). During the last 20 min the strips were subjected to continuous EFS (40 V, 1 ms, 0.5 Hz). After this equilibration period, superfusion was stopped and the preparations were incubated for 30 min with [^{3}H]-choline (5 µCi/ml) during which the tissues were stimulated electrically (40 V, 1 ms, 2 Hz) in order to label their cholinergic transmitter stores.

After the labelling procedure, the strips were superfused (2 ml/min) for 90 min with PSS to remove loosely bound radioactivity. From now on the PSS contained in addition 10^{-5} M hemicholinium-3 to prevent the re-uptake of choline, 10^{-5} M physostigmine to prevent the

hydrolysis of acetylcholine and, except in one series of experiments, 10^{-6} M atropine to prevent the auto-inhibition of acetylcholine release.

After the washout period, the strips were no longer superfused but the content of the organ bath (2 ml) was collected and replaced each 3 min. A total of 35 samples was collected. 1 ml of the samples was mixed with 4 ml of the scintillator containing solution Ultima Gold (Canberra Packard, USA). The strips were stimulated twice for 2 min (S_1 and S_2; 40 V, 1 ms, 4 Hz), at 13 min (S_1, 5th sample), and 73 min (S_2, 25th sample) after the end of the washout period. Tetrodotoxin (TTX), calcium-free medium, L-N^G-nitroarginine methyl ester (L-NAME), sodium nitroprusside (SNP), VIP and atropine were added 30 min (15th sample) before S_2. The α_2-adrenoceptor antagonist rauwolscine was added 30 min (15th sample) before S_2 and the α_2-adrenoceptor agonist, UK-14,304 was added 3 min (24th sample) before S_2. The 5-HT_4-receptor antagonist, SB204070 was added 36 min (13th sample) before S_2 and the 5-HT_4-receptor agonist, prucalopride was added 15 min (20th sample) before S_2. The α_2- and 5-HT_4-receptor antagonists and agonists were either given to the same tissue, or to separate tissues. Once added, drugs remained present until the end of the experiment. At the end of the experiment, tissues were blotted and weighed.

5.3.3. Measurement of radioactivity and separation by HPLC of radioactive compounds

Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Canberra Packard, USA). External standardization was used to correct for counting efficiency. Electrical stimulation induced an increase in tritium overflow, not only in samples 5 (S_1) and 25 (S_2) but also in the five samples after that with stimulation. The stimulation-induced increase in tritium overflow was calculated by subtracting basal tritium overflow. Basal tritium overflow during the period of enhanced tritium overflow was calculated by fitting a regression line through the values of the four samples just before stimulation and the values of the four samples starting from the sixth sample after stimulation.

The amount of [^{3}H]-acetylcholine, [^{3}H]-choline and [^{3}H]-phosphorylcholine in the samples was analysed by reverse phase HPLC (Bischoff Chromatography, Germany; Hyperchrome-HPLC-column, 250 x 4.6 mm, prepacked with HYPERSIL – ODS 5.0 µm). A 0.1 M phosphate buffer (pH 4.7) was used, containing methanol (8 vol %) and tetramethylammonium (0.2 mM). The flow was 0.5 ml/min and the effluent was collected in

one min fractions. This is a suitable method to separate the different components as we have demonstrated previously (Leclere & Lefebvre, 2001).

HPLC was performed on one sample before S_1 and S_2 (sample 3 before S_1 and sample 23 before S_2), and on the sample during stimulation (sample 5 and 25). 100 µl of the sample was injected into the HPLC; 27 fractions were collected, and each fraction was mixed with 2 ml of Ultima Gold. Fractions 7 to 12 contained the peaks of [^{3}H]-phosphorylcholine and [^{3}H]-choline and were taken together to calculate the amount of [^{3}H]-phosphorylcholine and [^{3}H]-choline, as both peaks could not be separated completely with the phosphate buffer we used. Fractions 14 to 25 were summed to calculate the amount of [^{3}H]-acetylcholine. The real amount of [^{3}H]-phosphorylcholine plus [^{3}H]-choline and of [^{3}H]-acetylcholine was calculated by subtracting the background counting. Background counting was calculated by fitting a regression line through the values of the first five fractions and fractions 26 and 27. Finally, the percentage of [^{3}H]-acetylcholine in each sample was calculated.

5.3.4. Drugs and radiochemicals

L-ascorbic acid, atropine sulphate, choline chloride, guanethidine sulphate, L-N^G-nitroarginine methyl ester, sodium nitroprusside and vasoactive intestinal polypeptide were obtained from Sigma (St. Louis, USA), hemicholinium-3-bromide from RBI (Natick, USA), methanol from Lab-Scan (Dublin, Ireland), [methyl-^{3}H]-choline chloride (2775 GBq/mmol) from NEN (Boston, USA), physostigmine salicylate from Federa (Brussels, Belgium), prucalopride (gift from Janssen Research Foundation, Beerse, Belgium), rauwolscine hydrochloride from Carl Roth KG (Karlsruhe, Germany), SB204070 ((1-butyl-4-piperidinyl)-methyl-8-amino-7-chloro1,4-benzodioxane-5-carboxylate HCl) (gift from SmithKline Beecham, Worthing, England), tetramethylammonium chloride from Merck-Schuchardt (Hohenbrunn, Germany), tetrodotoxin from Alomone Labs (Jerusalem, Israel) and UK-14,304 tartrate (5-bromo-6-[-2-imidazolin-2-ylamino]-quinoxaline) (gift from Pfizer, Sandwich, England).

Drugs were dissolved and diluted with distilled water. Stock solutions of 10^{-3} M TTX, 10^{-2} M prucalopride and 10^{-3} M SB204070 were kept frozen at −20°C. Dilutions were made the day of the experiment.

5.3.5. Data analysis

The ratios S_2/S_1 for total radioactivity (TR) and for tritiated acetylcholine were calculated. Experimental data are expressed as means ± s.e.mean and *n* refers to the number of tissues. Results were compared by the unpaired *t*-test or by ANOVA followed by a *t*-test corrected for multiple comparisons (Bonferroni procedure) when more than two responses had to be compared. *P* values of less than 0.05 were considered statistically significant.

5.4. Results

5.4.1. Control experiments

Tissues were electrically stimulated in PSS containing guanethidine ($4x10^{-6}$ M), hemicholinium-3 (10^{-5} M), physostigmine (10^{-5} M) and atropine (10^{-6} M) at 40 V, 1 ms, 4 Hz for 2 min after incubation with [^{3}H]-choline for 30 min. Field stimulation caused a clearcut increase in total radioactivity (TR) and 15 min were required after stimulation to re-establish the basal release of tritium. [^{3}H]-Acetylcholine could be detected when HPLC was used to separate the different components present in the samples. The mean amounts of TR, [^{3}H]-choline plus [^{3}H]-phosphorylcholine and [^{3}H]-acetylcholine before and during stimulation, as well as the percentage of [^{3}H]-acetylcholine released are given in Table 5.1. The S_2/S_1 ratios for TR and [^{3}H]-acetylcholine were respectively 0.72 ± 0.01 and 0.74 ± 0.02 (n = 23).

Table 5.1 Outflow of total radioactivity, [^{3}H]-choline plus [^{3}H]-phosphorylcholine and [^{3}H]-acetylcholine from the circular muscle of human proximal stomach.

	TR	Ch. + Ph.-Ch.	ACh	% ACh
Sample 3	46 500 ± 4 500	2 140 ± 200	380 ± 70	14 ± 2
S_1	1 556 700 ± 216 500***	6 550 ± 690***	28 640 ± 4 370***	77 ± 2***
Sample 23	28 700 ± 2 300	1 480 ± 150	280 ± 60	16 ± 3
S_2	1 093 300 ± 145 600###	3 080 ± 270###	19 080 ± 2 920###	83 ± 2###
S_2/S_1	0.72 ± 0.01		0.74 ± 0.02	

Radioactivity is expressed as dpm per g tissue (TR: Total radioactivity) and dpm per g tissue for 100 µl injected in the HPLC (Ch. + Ph.-Ch.: [^{3}H]-choline plus [^{3}H]-phosphorylcholine; ACh: [^{3}H]-acetylcholine). TR for S_1 and S_2 represents the sum of radioactivity above baseline in respectively sample 5 and sample 25 and the following five samples, while [^{3}H]-choline plus [^{3}H]-phosphorylcholine and [^{3}H]-acetylcholine for S_1 and S_2 represent the amount of these compounds detected by HPLC in 100 µl of respectively sample 5 and sample 25. Results are given as mean ± s.e.mean, n = 23. *** $P < 0.001$: Significantly different from values before S_1 (sample 3). ### $P < 0.001$: Significantly different from values before S_2 (sample 23).

TTX ($3x10^{-6}$ M; n = 3) or removal of extracellular calcium (n = 3) did not influence basal release of TR, but they nearly abolished the electrically-evoked tritium release as compared with control ($P < 0.001$). In control tissues (n = 3), the S_2/S_1 ratios for release of TR and [^{3}H]-acetylcholine were 0.71 ± 0.06 and 0.69 ± 0.03, respectively. After superfusion with TTX or calcium-free medium, the S_2/S_1 ratios for TR were respectively 0.08 ± 0.04 and 0.05 ± 0.02, while those for [^{3}H]-acetylcholine were respectively 0.01 ± 0.02 and 0.00 ± 0.02.

5.4.2. The effects of L-N^G-nitroarginine methyl ester, sodium nitroprusside and VIP

The NO synthase inhibitor L-NAME ($3x10^{-4}$ M; n = 6) or the NO donor SNP (10^{-5} M; n = 5), when added 30 min before the second stimulation, had no influence on basal release of TR. L-NAME or SNP had also no effect on the electrically-evoked increase of TR or [^{3}H]-acetylcholine released (Table 5.2). Similarly, VIP (10^{-7} M; n = 5), added 30 min before S_2, was without effect on the basal release of TR and on the electrically-evoked increase in release of TR or [^{3}H]-acetylcholine (Table 5.2).

5.4.3. The effects of UK-14,304 and rauwolscine

The α_2-adrenoceptor antagonist rauwolscine ($2x10^{-6}$ M; n = 9) did not alter the basal efflux of TR, nor had it any effect *per se* on the S_2/S_1 ratio for TR (S_2/S_1 ratios for control and rauwolscine: 0.66 ± 0.04 and 0.66 ± 0.05, respectively, $P > 0.05$; n = 4).

Table 5.2 Influence of a NOS inhibitor, a NO donor and VIP on the ratio (S_2/S_1) of the efflux of total radioactivity and [^{3}H]-acetylcholine induced by two periods of stimulation.

Drugs added before S_2	TR	ACh	*n*
None	0.71 ± 0.03	0.77 ± 0.04	6
L-NAME ($3x10^{-4}$ M)	0.72 ± 0.03	0.74 ± 0.10	6
None	0.70 ± 0.04	0.78 ± 0.04	5
SNP (10^{-5} M)	0.69 ± 0.03	0.75 ± 0.05	5
None	0.74 ± 0.03	0.76 ± 0.02	5
VIP (10^{-7} M)	0.79 ± 0.02	0.81 ± 0.03	5

After incubation with [^{3}H]-choline and washout, tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min) and the content of the organ bath (2 ml) was collected every 3 min. The electrically-evoked efflux by S_2 is expressed as a ratio of that by S_1. HPLC was performed to calculate the S_2/S_1 ratio for [^{3}H]-acetylcholine. L-N^G-nitroarginine methyl ester (L-NAME), sodium nitroprusside (SNP) or vasoactive intestinal polypeptide (VIP) were added 30 min before S_2. TR: total radioactivity; ACh: [^{3}H]-acetylcholine. Mean ± s.e.mean. *n* refers to number of tissues used.

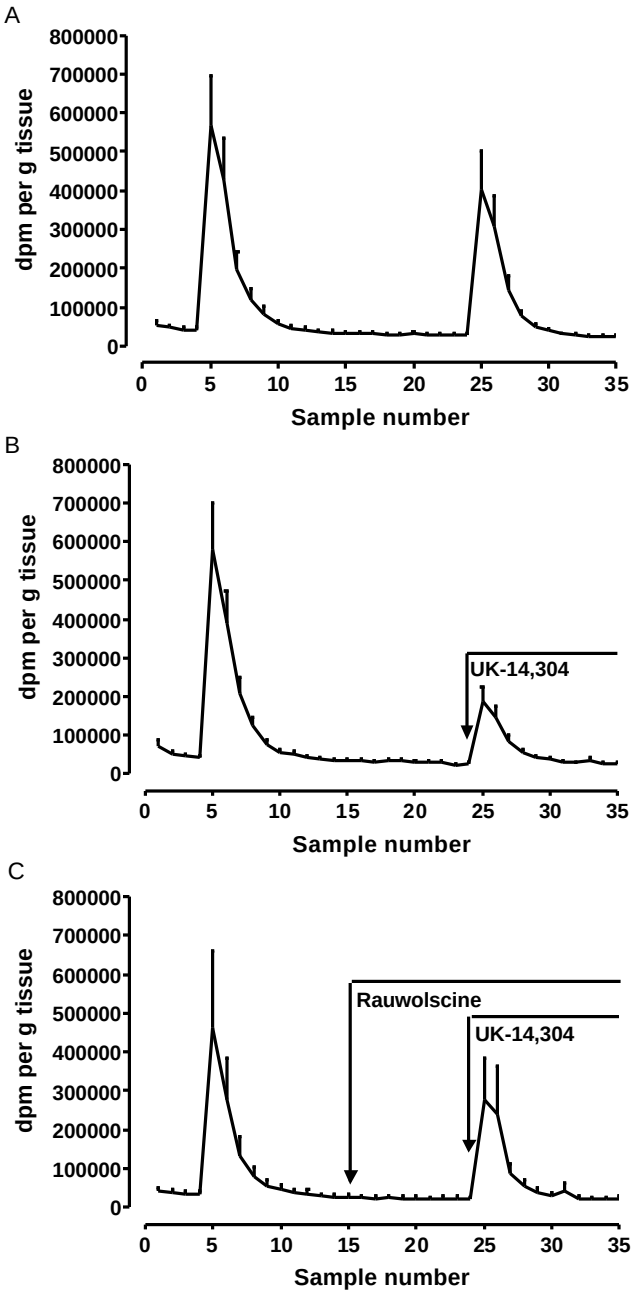

Figure 5.1 Effects of UK-14,304 and UK-14,304 in the presence of rauwolscine on the electrically-evoked release of total radioactivity (TR) from preparations of human proximal stomach pre-incubated with [^{3}H]-choline. Tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min), and the content of the organ bath (2 ml) was collected in 3 min samples for TR. **A.** TR outflow of control experiments (n = 6). **B.** Release of TR when UK-14,304 (10^{-5} M) was added 3 min before S_2 (n = 6). **C.** Release of TR when rauwolscine ($2x10^{-6}$ M) was added 30 min and UK-14,304 (10^{-5} M) 3 min before S_2 (n = 5). The results are given as mean ± s.e.mean.

The selective α_2-adrenoceptor agonist UK-14,304 (10^{-5} M), added 3 min before the second stimulation period, did not alter the basal efflux of TR (n = 6). However, UK-14,304 significantly reduced the amount of TR and [^{3}H]-acetylcholine released upon electrical stimulation. In control tissues, the S_2/S_1 ratios for release of TR and [^{3}H]-

acetylcholine were 0.72 ± 0.02 and 0.70 ± 0.03 respectively (Figure 5.1A), while in the presence of UK-14,304 the ratios were 0.33 ± 0.03 and 0.31 ± 0.04 ($P < 0.001$; $n = 6$; Figure 5.1B). Rauwolscine prevented the inhibition of the electrically-evoked release of TR and [^{3}H]-acetylcholine by UK-14,304 (S_2/S_1 ratios for TR and [^{3}H]-acetylcholine in the presence of rauwolscine: 0.67 ± 0.06 and 0.58 ± 0.04 respectively, $P > 0.05$ compared with control, $n = 5$; Figure 5.1C).

5.4.4. The effects of prucalopride and SB204070

The 5-HT$_4$-receptor antagonist SB204070 (10^{-9} M; $n = 8$) did not alter the basal release of TR, nor did SB204070 influence the S_2/S_1 ratio for TR *versus* control (0.64 ± 0.06 and 0.66 ± 0.04, respectively, $P > 0.05$; $n = 4$).

The selective 5-HT$_4$-receptor agonist prucalopride ($3x10^{-7}$ M) did not alter the basal efflux of TR. However, prucalopride significantly enhanced the amount of TR ($P < 0.001$) and [^{3}H]-acetylcholine ($P < 0.01$) released upon electrical stimulation ($n = 6$; Figure 5.2). When SB204070 was added before prucalopride, it completely prevented the increase of the electrically-evoked release of TR and [^{3}H]-acetylcholine by prucalopride ($P > 0.05$; $n = 4$; Figure 5.2).

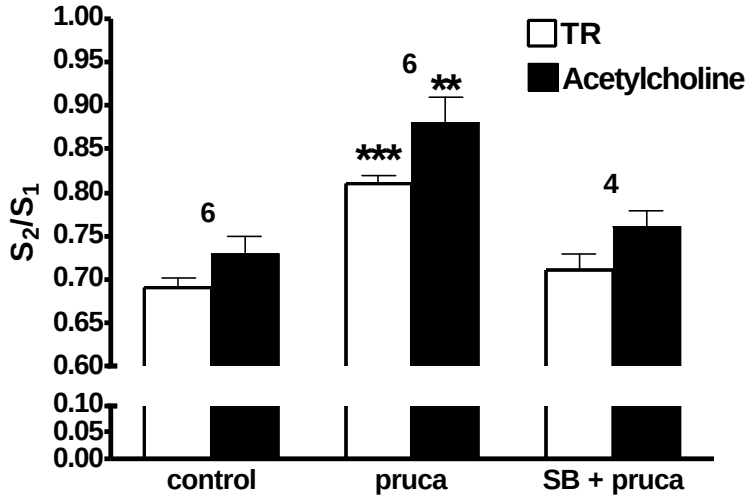

Figure 5.2 Effects of prucalopride (pruca) and prucalopride in the presence of SB204070 (SB) on the electrically-evoked release of total radioactivity (TR) and [^{3}H]-acetylcholine from preparations of human proximal stomach pre-incubated with [^{3}H]-choline. Tissues were stimulated twice (S_1 and S_2: 40 V, 1 ms, 4 Hz, 2 min); SB204070 (10^{-9} M) was added 36 min and prucalopride ($3x10^{-7}$ M) 15 min before S_2. The electrically-evoked efflux by S_2 is expressed as a ratio of that by S_1. HPLC was performed to calculate the S_2/S_1 ratio for [^{3}H]-acetylcholine. Each column represents the mean ± s.e.mean. ** $P < 0.01$; *** $P < 0.001$: Significantly different from control. Numbers above the columns refer to number of tissues used.

5.4.5. The effect of atropine

Until now, all experiments were performed in the presence of the muscarinic antagonist atropine. To study whether the released acetylcholine is able to influence its own release, a series of experiments was done in the absence of atropine. In the absence of atropine, the mean basal release of TR before S_1 (sample 3) was 54 200 ± 14 600 dpm per g tissue (n = 6), which is not significantly different from the basal release in the presence of atropine. However, the mean increase in TR released during the first stimulation was 563 600 ± 153 400 dpm per g tissue, a significant decrease in comparison to tissues stimulated in the presence of atropine (1 556 700 ± 216 500 dpm per g tissue, see Table 5.1, $P < 0.05$), suggesting that acetylcholine inhibits its own release. This was confirmed by the observation that atropine (10^{-6} M), added 30 min before S_2, significantly increased the S_2/S_1 ratios for both TR and [^{3}H]-acetylcholine released upon electrical stimulation, without having an effect on basal release. In control tissues, the S_2/S_1 ratios for release of TR and [^{3}H]-acetylcholine were 0.82 ± 0.05 (n = 6) and 0.92 ± 0.04 (n = 5) respectively, while the ratios were 2.03 ± 0.22 ($P < 0.001$; n = 6) and 2.70 ± 0.52 ($P < 0.01$; n = 5) when atropine was added between S_1 and S_2.

5.5. Discussion

This study investigated whether the release of acetylcholine in the human proximal stomach can be influenced by NO or VIP, or *via* presynaptic muscarinic, α_2- or 5-HT_4-receptors, as assessed by direct measurement of [^{3}H]-acetylcholine release. Experiments were conducted on preparations which had been incubated with [^{3}H]-choline to incorporate [^{3}H]-acetylcholine into the cholinergic transmitter stores.

5.5.1. Control experiments

Incubation of the human proximal stomach with [^{3}H]-choline resulted in the synthesis of [^{3}H]-acetylcholine, that was released during field stimulation. The field-stimulated release of TR and [^{3}H]-acetylcholine was prevented by TTX or by the removal of extracellular calcium, indicating a neuronal release dependent upon respectively, the opening of sodium channels, and the presence of calcium in the external medium. Basal release of TR was not influenced in the presence of TTX or the absence of extracellular calcium, indicating a low

degree of spontaneous activity of the cholinergic neurones during rest. This implies a low exchange of newly synthesised [^{3}H]-acetylcholine against unlabelled acetylcholine, suggesting that electrical stimulation during labelling is necessary, as it is in the rat myenteric plexus and the pig gastric fundus (Wessler & Werhand, 1990; Leclere & Lefebvre, 2001).

Electrical stimulation not only caused an increase in the release of [^{3}H]-acetylcholine, but also a moderate increase in the outflow of [^{3}H]-phosphorylcholine and [^{3}H]-choline. Our results are in contrast with results in the guinea-pig and rat myenteric plexus, where electrical field stimulation only caused an increase in [^{3}H]-acetylcholine release (Wessler & Werhand, 1990; Hebeiβ & Kilbinger, 1996), but are in agreement with results in the canine ileum, pig gastric fundus and rat and guinea-pig trachea (Wessler *et al.*, 1990, 1991; Hryhorenko *et al.*, 1994; Leclere & Lefebvre, 2001). As the S_2/S_1 ratio for [^{3}H]-acetylcholine was systematically similar to that for TR, it will no longer be necessary to separate all radioactive components in future experiments with this tissue as the results of TR reflect those of [^{3}H]-acetylcholine.

5.5.2. Presynaptic modulation of acetylcholine release

5.5.2.a. Acetylcholine

In this type of experiments, the presence of an acetylcholinesterase inhibitor (physostigmine) is required to be able to determine the amount of [^{3}H]-acetylcholine released. Our previous experiments in the pig gastric fundus illustrated that the process of auto-inhibition of acetylcholine release *via* stimulation of presynaptic muscarinic auto-receptors on the cholinergic neurones is increased in the presence of physostigmine. This auto-inhibition was prevented by adding atropine together with physostigmine (Leclere & Lefebvre, 2001), and this condition was also used in the actual human proximal stomach experiments. The presence of inhibitory presynaptic muscarinic auto-receptors in the human proximal stomach was confirmed in the experiments in the absence of atropine in the basal medium since: (1) when only physostigmine was present, about three times less tritium was liberated during electrical stimulation; (2) the ratio S_2/S_1 for TR and [^{3}H]-acetylcholine was significantly increased when atropine was added before S_2 compared to control strips in the presence of physostigmine alone. The presence of presynaptic muscarinic auto-receptors on the cholinergic neurones of human proximal stomach is in agreement with the general concept

that presynaptic muscarinic receptors inhibit the release of acetylcholine from peripheral ends of parasympathetic nerve fibres in tissues of different species (see review Starke *et al.*, 1989).

5.5.2.b. NO and VIP

In several species and tissues it has been demonstrated that NO can act presynaptically on cholinergic neurones to enhance the basal release of acetylcholine and to inhibit the electrically-evoked release of acetylcholine (Kilbinger, 1996). Functional experiments in the human gastric fundus led to the proposal that endogenous NO might tonically inhibit the release of acetylcholine (Tonini *et al.*, 2000). From functional studies, however, it is not possible to determine with certainty the site(s) of action of NO (presynaptic inhibition of acetylcholine release *versus* postsynaptic functional antagonism). For this reason, the effects of a NO synthase inhibitor and a NO donor on [^{3}H]-acetylcholine release in the human proximal stomach were determined. Blockade of NO synthase, or addition of SNP did not significantly affect the basal release of TR or the electrically-evoked overflow of TR and [^{3}H]-acetylcholine, suggesting that NO does not modulate acetylcholine release in the human proximal stomach. This is in agreement with the findings in other species and tissues where NO donors and NO synthase inhibitors do not modify [^{3}H]-acetylcholine release in either tracheal or intestinal preparations (Brave *et al.*, 1991; Ward *et al.*, 1993, 1996; Milenov & Kalfin, 1996; Rae *et al.*, 1998; Leclere & Lefebvre, 2001). Still, as the strips are cut in the direction of the circular muscle layer, it cannot be excluded that longitudinally directed interneurones and/or sensory neurones are not fully assessed; modulation of acetylcholine released by NO might still be present at this level.

As VIP is co-localised with NO in the majority of the myenteric neurones in the human gastric fundus, and VIP is released during electrical field stimulation (Tonini *et al.*, 2000), the effect of VIP on the electrically-evoked release of acetylcholine was investigated. The concept that VIP can inhibit cholinergic neurotransmission *via* a presynaptic mechanism has indeed been proposed for gastrointestinal and respiratory tissue (Hakoda & Ito, 1990; Milenov *et al.*, 1991; Baccari *et al.*, 1994). VIP did not significantly affect the basal release of TR or electrically-evoked overflow of TR and [^{3}H]-acetylcholine, suggesting that VIP does not modulate [^{3}H]-acetylcholine release in human proximal stomach. This is in agreement with observations in various gastrointestinal and respiratory tissues (Lefebvre *et al.*, 1992; Ward *et al.*, 1993; Sekizawa *et al.*1993), although modulation of acetylcholine released by VIP might still be present at the level of the longitudinally directed interneurones and/or

sensory neurones. Indeed, it has been demonstrated that PACAP and VIP can stimulate the spontaneous and inhibit the electrically-evoked release of [^{3}H]-acetylcholine of guinea-pig longitudinal muscle myenteric plexus preparations (Katsoulis *et al.*, 1993).

5.5.2.c. Presynaptic α_2-adrenoceptors

It has already been shown that presynaptic inhibitory α_2-adrenoceptors are present on cholinergic neurones in various gastrointestinal and other tissues (see introduction; for review De Ponti *et al.*, 1996). In the actual study, the α_2-adrenoceptor agonist UK-14,304 significantly reduced the stimulation-induced efflux of TR and [^{3}H]-acetylcholine in the human proximal stomach, as it did in guinea-pig ileum and pig gastric fundus (Funk *et al.*, 1995; Leclere & Lefebvre, 2001). The incubation period of 3 min for UK-14,304 is sufficient as increasing this period to 30 min did not increase the inhibitory effect of UK-14,304 (results not shown). The inhibition of the stimulated overflow produced by UK-14,304 was antagonised by rauwolscine, a selective α_2-adrenoceptor antagonist (Weitzell *et al.*, 1979), indicating that cholinergic nerves of the human proximal stomach are endowed with α_2-adrenoceptors, causing inhibition of acetylcholine release. This corresponds with the general idea that endogenous noradrenaline is able to inhibit non-sphincteric muscle in the gastrointestinal tract by inhibition of acetylcholine release from the cholinergic motor neurones *via* presynaptic α_2-adrenoceptors (McIntyre & Thompson, 1992). Whether endogenous noradrenaline is able to influence acetylcholine release in the human proximal stomach within the experimental conditions was not assessed, as guanethidine was continuously present in the PSS, preventing the release of endogenous noradrenaline.

5.5.2.d. Presynaptic 5-HT$_4$-receptors

Stimulation of gastrointestinal 5-HT$_4$-receptors induces contraction or relaxation depending on the tissue and species studied. Contractile responses are generally ascribed to 5-HT$_4$-receptors localized on cholinergic neurones, facilitating acetylcholine release (see e.g. Elswood *et al.*, 1991; Kilbinger & Wolf, 1992; Briejer & Schuurkes, 1996) while the relaxant responses are due to 5-HT$_4$-receptors localized on smooth muscle (see e.g. Tam *et al.*, 1995; Prins *et al.*, 2000). In the stomach, facilitatory 5-HT$_4$-receptors on cholinergic neurones are present in the guinea-pig (Buchheit & Buhl, 1994; Matsuyama *et al.*, 1996) and rat (Amemiya

et al., 1996). Whereas nervous 5-HT_4-receptors seem present in the rat gastric fundus (Amemiya *et al.*, 1996), a recent study in the guinea-pig stomach suggested a regional distribution of the nervous 5-HT_4-receptors, being present in the corpus and antrum but absent in the fundus (Takada *et al.*, 1999). In this study, we assessed the possible presence of facilitatory 5-HT_4-receptors on the cholinergic nerves by use of the selective 5-HT_4-receptor agonist prucalopride (Briejer *et al.*, 1998). Prucalopride significantly increased the stimulation-induced efflux of TR and [^{3}H]-acetylcholine in the human proximal stomach. This increase was completely antagonised by SB204070, a selective 5-HT_4-receptor antagonist (Wardle *et al.*, 1994). This indicates that cholinergic nerves of the human proximal stomach are endowed with 5-HT_4-receptors stimulating acetylcholine release. A stimulatory effect of gastroprokinetic agents such as cisapride can thus also be expected at the level of the human proximal stomach. As SB204070 did not influence the S_2/S_1 ratio for TR, it is unlikely that electrical stimulation causes the release of endogenous 5-HT which might act on 5-HT_4-receptors to enhance [^{3}H]-acetylcholine release.

In conclusion, the data provided by this study indicate that measurement of tritium release after incubation with [^{3}H]-choline can be used to reflect endogenous acetylcholine release in response to cholinergic neuron stimulation in the human proximal stomach. The results indicated the presence of presynaptic inhibitory α_2-adrenoceptors and muscarinic auto-receptors, and excitatory 5-HT_4-receptors. No evidence for the modulation of acetylcholine release by NO or VIP was obtained.

5.6. References

ABRAHAMSSON, H. (1986). Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch. Int. Pharmacodyn.*, **280** (suppl.), 50-61

AMEMIYA, N., HATTA, S., TAKEMURA, H. & OHSHIKA, H. (1996). Characterization of the contractile response induced by 5-methoxytryptamine in rat stomach fundus strips. *Eur. J. Pharmacol.*, **318**, 403-409

BACCARI, M.C., BERTINI, M. & CALAMAI, F. (1993) Effects of L-N^G-nitro arginine on cholinergic transmission in the gastric muscle of the rabbit. *Neuroreport*, **4**, 1102-1104

BACCARI, M.C., CALAMAI, F., STADERINI, G. (1994). Modulation of cholinergic transmission by nitric oxide, VIP and ATP in the gastric muscle. *Neuroreport*, **5**, 905-908

BRAVE, S.R., HOBBS, A.J., GIBSON, A. & TUCKER, J.F. (1991). The influence of L-N^G-nitro-arginine on field stimulation induced contractions and acetylcholine release in guinea pig isolated tracheal smooth muscle. *Biochem. Biophys. Res. Comm.*, **179**, 1017-1022

BRIEJER, M.R., MEULEMANS, A.L., BOSMANS, J.-P., VAN DAELE, P. & SCHUURKES, J.A.J. (1998). In vitro pharmacology of the novel enterokinetic R093877. *Gastroenterology*, **112**, A704

BRIEJER, M.R. & SCHUURKES, J.A.J. (1996). 5-HT_3 and 5-HT_4 receptors and cholinergic and tachykininergic neurotransmission in the guinea-pig proximal colon. *Eur. J. Pharmacol.*, **308**, 173-180

BUCHHEIT, K. & BUHL, T. (1994). Stimulant effects of 5-hydroxytryptamine on guinea-pig stomach preparations in vitro. *Eur. J. Pharmacol.*, **262**, 91-97

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.*, **69**, 59-78

ELSWOOD, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Identification of putative 5-HT_4 receptors in guinea-pig ascending colon. *Eur. J. Pharmacol.*, **196**, 149-155

FUNK, L., TRENDELENBURG,A.-U., LIMBERGER, N. & STARKE, K. (1995). Subclassification of presynaptic α_2-adrenoceptors: α_{2D}-autoreceptors and α_{2D}-adrenoceptors modulating release of acetylcholine in guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352**, 58-66

HAKODA, H. & ITO, Y. (1990). Modulation of cholinergic neurotransmission by the peptide VIP, VIP antiserum and VIP antagonists in dog and cat trachea. *J. Physiol.*, **428**, 133-154

HEBEIβ, K. & KILBINGER, H. (1996). Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. *Br. J. Pharmacol.*, **118**, 2073-2078

HRYHORENKO, L.M., WOSKOWSKA, Z. & FOX-THRELKELD, J.-A.E.T. (1994). Nitric oxide (NO) inhibits release of acetylcholine from nerves of isolated circular muscle of the canine ileum : relationship to motility and release of nitric oxide. *J. Pharmacol. Exp. Ther.*, **271**, 918-926

JANSSON, G., LISANDER, B. (1969). On adrenergic influences on gastric motility in chronically vagotomized cats. *Acta Physiol. Scand.*, **76**, 463-471

KATSOULIS, S., CLEMENS, A., SCHWÖRER, H., CREUTZFELDT, W. & SCHMIDT, W.E. (1993). PACAP is a stimulator of neurogenic contraction in guinea pig ileum. *Am. J. Physiol.*, **265**, G295-G302

KELLY, K.A. (1980). Gastric emptying of liquids and solids: roles of proximal and distal stomach. *Am. J. Physiol.*, **239**, G71-G76

KILBINGER, H. (1996). Modulation of acetylcholine release by nitric oxide. *Progress in Brain Res.*, **109**, 219-224

KILBINGER, H. & WOLF, D. (1992). Effects of 5-HT_4 receptor stimulation on basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **345**, 270-275

LECLERE, P.G. & LEFEBVRE, R.A. (2001). Influence of nitric oxide donors and of the α_2-agonist UK-14,304 on acetylcholine release in the pig gastric fundus. *Neuropharmacology*, **40**, 270-278

LEFEBVRE, R.A. (1993). Non-adrenergic non-cholinergic neurotransmission in the proximal stomach. *Gen. Pharmacol.*, **24**, 257-266

LEFEBVRE, R.A., DE VRIESE, A. & SMITS, G.J.M. (1992). Influence of vasoactive intestinal polypeptide and N^G-nitro-L-arginine methyl ester on cholinergic neurotransmittion in the rat gastric fundus. *Eur. J. Pharmacol.*, **221**, 235-242

LEFEBVRE, R.A., WILLEMS, J.L. & BOGAERT, M.G. (1984). Inhibitory effect of dopamine on canine gastric fundus. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **326**, 22-28

MACDONALD, A., KELLY, J. & DETTMAR, P.W. (1990). Pre- and post-junctional α-adrenoceptor-mediated responses in the rat gastric fundus in vitro. *J. Pharm. Pharmacol.*, **42**, 752-757

MATSUYAMA, S., SAKIYAMA, H., NEI, K. & TANAKA, C. (1996). Identification of putative 5-hydroxytryptamine$_4$ (5-HT_4) receptors in guinea pig stomach: the effect of TKS159, a novel agonist, on gastric motility and acetylcholine release. *J. Pharmacol. Exp. Ther.*, **276**, 989-995

MCINTYRE, A.S. & THOMPSON, D.G. (1992). Review article: adrenergic control of motor and secretory function in the gastrointestinal tract. *Aliment. Pharmacol. Ther.*, **6**, 125-142

MILENOV, K. & KALFIN, R. (1996). Cholinergic-nitrergic interactions in the guinea-pig gastric fundus. *Neuropeptides*, **30**, 365-371

MILENOV, K., KALFIN, R. & MANDREK, K. (1991). Effect of vasoactive intestinal peptide (VIP) on the mechanical activity and [^{3}H] acetylcholine release in guinea-pig gastric muscle. *Acta Physiol. Pharmacol. Bulg.*, **17**, 13-18

OGISHIMA, M., KAIBARA, M., UEKI, S., KURIMOTO, T. & TANIYAMA, K. (2000). Z-338 facilitates acetylcholine release from enteric neurons due to blockade of muscarinic autoreceptors in guinea pig stomach. *J. Pharmacol. Exp. Ther.*, **294**, 33-37

PARKMAN, H.P., TRATE, D.M., KNIGHT, L.C., BROWN, K.L., MAURER, A.H. & FISHER, R.S. (1999). Cholinergic effects on human gastric motility. *Gut*, **45**, 346-354

PATERSON, C.A., ANVARI, M., TOUGAS, G. & HUIZINGA, J.D. (2000). Nitrergic and cholinergic vagal pathways involved in the regulation of canine proximal gastric tone: an *in vivo* study. *Neurogastroenterol. Mot.*, **12**, 301-306

PRINS, N.H., SHANKLEY, N.P., WELSH, N.J., BRIEJER, M.R., LEFEBVRE, R.A., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (2000). An improved *in vitro* bioassay for the study of 5-HT_4 receptors in the human isolated large intestinal circular muscle. *Br. J. Pharmacol.*, **129**, 1601-1608

RAE, M.G., KHOYI, M.A. & KEEF, K.D. (1998). Modulation of cholinergic neuromuscular transmission by nitric oxide in canine colonic circular smooth muscle. *Am. J. Physiol.*, **275**, G1324-G1332

SEKIZAWA, K., FUKUSHIMA, T., IKARASHI, Y., MARUYAMA, Y. & SASAKI, H. (1993). The role of nitric oxide in cholinergic neurotransmission in rat trachea. *Br. J. Pharmacol.*, **110**, 816-820

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of transmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 864-989

TAKADA, K., SAKURAI-YAMASHITA, Y., YAMASHITA, K., KAIBARA, M., HAMADA, Y., NAKANE, Y., HIOKI, K. & TANIYAMA, K. (1999). Regional difference in correlation of 5-HT_4 receptor distribution with cholinergic transmission in the guinea pig stomach. *Eur. J. Pharmacol.*, **374**, 489-494

TAM, F.S.F., HILLIER, K., BUNCE, K.T. & GROSSMAN, C. (1995). Differences in response to 5-HT_4 receptor agonists and antagonists of the 5-HT_4-like receptor in human colon circular smooth muscle. *Br. J. Pharmacol.*, **115**, 172-176

TONINI, M., DE GIORGIO, R., DE PONTI, F., STERNINI, C., SPELTA, V., DIONIGI, P., BARBARA, G., STANGHELLINI, V. & CORINALDESI, R. (2000). Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations. *Br. J. Pharmacol.*, **129**, 12-20

VALENZUELA, J.E. & LIU, D.P. (1982). The effect of variations in intragastric pressure and gastric emptying of a saline meal in humans. *Scand. J. Gastroenterol.*, **17**, 293-296

WARD, J.K., BELVISI, M.G., FOX, A.J., MIURA, M., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. *J. Clin. Invest.*, **92**, 736-743

WARD, S.M., DALZIEL, H.H., KHOYI, M.A., WESTFALL, A.S., SANDERS, K.M. & WESTFALL, D.P. (1996). Hyperpolarization and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli. *Br. J. Pharmacol.*, **118**, 49-56

WARDLE, K.A., ELLIS, E.S., BAXTER, G.S., KENNETT, G.A., GASTER, L.M. & SANGER, G.J. (1994). The effects of SB204070, a highly potent and selective 5-HT_4 receptor antagonist, on guinea-pig distal colon. *Br. J. Pharmacol.*, **112**, 789-794

WEITZELL, R., TANAKA, T. & STARKE, K. (1979). Pre- and postsynaptic effects of yohimbine stereoisomers on noradrenergic transmission in the pulmonary artery of the rabbit. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **308**, 127-136

WESSLER, I., HELLWIG, D. & RACKÉ, K. (1990). Epithelium-derived inhibition of [^{3}H]acetylcholine release from the isolated guinea-pig trachea. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **342**, 387-393

WESSLER, I., KLEIN, A., POHAN, D., MACLAGAN, J. & RACKÉ, K. (1991). Release of [^{3}H]acetylcholine from the isolated rat or guinea-pig trachea evoked by preganglionic nerve stimulation; a comparison with transmural stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **344**, 403-411

WESSLER, I. & WERHAND, J. (1990). Evaluation by reverse phase HPLC of [^{3}H]acetylcholine release evoked from the myenteric plexus of the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 510-516

CHAPTER 6

5-HT_4-RECEPTORS LOCATED ON CHOLINERGIC NERVES AS WELL AS ON SMOOTH MUSCLE CELLS INVOLVED IN CONTRACTILITY OF HUMAN COLON CIRCULAR MUSCLE

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CHAPTER 6

5-HT$_4$-RECEPTORS LOCATED ON CHOLINERGIC NERVES AS WELL AS ON SMOOTH MUSCLE CELLS INVOLVED IN CONTRACTILITY OF HUMAN COLON CIRCULAR MUSCLE

6.1. Summary

5-HT$_4$-receptor agonists promote colonic propulsion. The alteration of circular muscle (CM) motility underlying this involves inhibition of contractility *via* smooth muscle 5-HT$_4$-receptors and proximal colonic motility stimulation, the mechanism of the latter not having been characterised. To identify and characterise a 5-HT$_4$-receptor-mediated stimulation of human colon CM contractile activity, 5-HT$_4$-receptor ligands were tested on electrical field stimulation (EFS)-induced contractions of human colonic muscle strips cut in the circular direction (called 'whole tissue' strips). Additionally, after incubation of tissues with [^{3}H]-choline these compounds were tested on EFS-induced release of tritium in whole tissue strips and in 'isolated' CM strips, obtained by superficial cutting in the circular muscle layer. Tetrodotoxin (TTX) and atropine blocked EFS-induced contractions of whole tissue CM strips. Prucalopride ($3x10^{-7}$ M) evoked a heterogenous response on EFS-induced contraction, ranging from inhibition (most frequently observed) to enhancement. In the release experiments, EFS-induced tritium efflux was blocked by TTX. Prucalopride increased EFS-induced tritium and [^{3}H]-acetylcholine efflux in whole tissue as well as in isolated CM strips. All effects of prucalopride were antagonised by the selective 5-HT$_4$-receptor antagonist GR113808. The results obtained indicate the presence of 5-HT$_4$-receptors on cholinergic nerves within the CM of human colon.

6.2. Introduction

5-HT$_4$-receptors have been described to be abundantly expressed in the gastrointestinal tract, mediating various aspects of gut motor function (Hegde & Eglen, 1996). Although most pharmacological studies were performed with intact rodents or rodent isolated tissues, dogs and humans are endowed with these receptors as well. This was demonstrated in recent *in vivo* and *in vitro* studies. In humans, 5-HT$_4$-receptor agonists have been demonstrated to stimulate whole gut transit, as well as colonic transit (Emmanuel *et al.*, 1998; Bouras *et al.*,

1999). Colonic contractility patterns were influenced by the 5-HT_4-receptor agonist prucalopride in conscious dogs, equipped with circularly orientated force transducers on the various regions of large intestine (Briejer *et al.*, 2001b) and the change of colonic motility was often associated with increased stool frequency. Prucalopride stimulated proximal, and inhibited distal colonic motility, and reduced the time to the first giant migrating contraction (GMC). GMCs are held responsible for mass movements over longer distances of large intestine (Karaus & Sarna, 1987). We have attempted to establish correlates of these *in vivo* actions by means of isolated tissue studies.

In the longitudinal muscle layer of both canine large intestine (from ascending to descending colon) and human large intestine (from ascending colon to rectum), 5-HT_4-receptors on cholinergic nerves mediate facilitation of cholinergic neurotransmission resulting in enhanced longitudinal muscle contractility (Prins *et al.*, 2000a). In canine large intestine circular muscle (CM), smooth muscle 5-HT_4-receptors mediate relaxation, a phenomenon that gradually increases in efficiency going from the ascending colon to the rectum (Prins *et al.*, 1999). In human large intestine CM, 5-HT_4-receptors located on smooth muscle mediate relaxation, irrespective of the region observed (Prins *et al.*, 2000b). This inhibitory action on circular muscle tone demonstrated *in vitro* may explain the distally increasing circular muscle inhibition observed with prucalopride in dog large intestine *in vivo* (Briejer *et al.*, 2001b). Although the effects of 5-HT_4-receptor agonists on human colonic motility *in vivo* have not been documented, it can be anticipated from the inhibitory 5-HT_4-receptors on colon CM that also in humans, activation of 5-HT_4-receptors will be associated with reduction of large intestine luminal resistance. However, the stimulation of human colonic transit by 5-HT_4-receptor agonists cannot be triggered by inhibition of circular muscle activity alone. It remains thus to be investigated what mechanism underlies the 5-HT_4-receptor-mediated stimulation of colonic motility in the circular muscle. We hypothesised that in human colon circular muscle, in addition to the presence of inhibitory smooth muscle 5-HT_4-receptors, excitatory 5-HT_4-receptors are present on cholinergic neurones.

We set out to investigate this *via* two experimental approaches. First, in classical organ bath experiments we tested the effect of a 5-HT_4-receptor agonist and antagonist on electrical field stimulation (EFS)-induced contractions of strips from human large intestinal circular muscle. Second, we assessed the effect of these ligands on EFS-triggered release of tritiated acetylcholine from those muscle strips.

6.3. Methods

6.3.1. Tissue preparation

With the approval of the local ethics committee, human large intestine specimens (6 ascending colon, 2 transverse colon, 1 descending colon and 11 sigmoid colon) were obtained during colectomy surgery for colonic cancer.

6.3.1.a. Dissection and preparation of muscle strips

The segments were cut open along the longitudinal axis. Luminal contents were rinsed out with Krebs-Henseleit solution (composition in mM: glucose 11.1, $CaCl_2$ 2.51, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.69 and NaCl 118) and the mucosa and adhering mesentery were removed. The tissue was stored in fresh solution at 4 °C to be used the next day.

There were two ways of dissection of CM strips. The first one was obtained by cutting through the entire wall in the circular direction. This conventional dissection method yielded CM strips that contained circular muscle, some longitudinal muscle, and the intermediate myenteric plexus. These strips were called 'whole tissue' CM strips and were used for both organ bath experiments and release experiments. Using a light microscope, the other type of CM strips was obtained by superficial cutting in the circular muscle layer with a pair of curved scissors. This provided CM strips that did not contain longitudinal muscle and they were called for future reference 'isolated' CM strips. Isolated CM strips were used for release experiments only. This dissection procedure was performed to evaluate the possible contribution of excitatory 5-HT_4-receptors on cholinergic neurones in the longitudinal muscle layer (Prins *et al.*, 2000a). We were aided in this dissection procedure by the use of intertaenial tissue of human specimens, in which the longitudinal muscle layer is clearly less pronounced.

6.3.2. Contractility study

Only 'whole tissue' muscle strips (measuring about 2-3 cm in length and 2-3 mm in width) were used to study contractility as isolated CM strips only showed very weak

contractions to electrical stimulation. The strips were anchored to organ bath hooks between platinum-wire electrodes (40 mm length, 0.5 mm width) and suspended in a classical organ bath set-up for isotonic measurement (2g load). The 20 ml organ baths were filled with Krebs-Henseleit solution, kept at 37 °C and gassed with carbogen (95% O_2, 5% CO_2). Electrical field stimulation (EFS) was applied using stimulation equipment made at the Janssen Research Foundation.

6.3.2.a. Experimental protocol of contractility study

After a 30 min period of stabilisation, the strips were contracted with carbachol (10^{-5} M) to test their viability and responsiveness. After wash-out, N^G-nitro-L-arginine (L-NNA; 10^{-4} M) was added to the organ bath solution in order to prevent relaxation due to EFS-induced release of nitric oxide. After 30 min of incubation, the muscle strips were electrically stimulated (initial parameters: 1 ms pulses in trains of 10 s, at an interval of 3 min, 20 V at 12 Hz). Each pulse train resulted in a contraction, and after 4-5 consecutive pulse trains, contractions were reproducible. The voltage was then reduced until reproducible contractions with an amplitude approximating 30-50% of the contraction observed at 20 V was obtained. Stimulation at this voltage was continued for at least 15 min, followed by addition of the selective 5-HT_4 receptor antagonist, GR113808 or solvent, which, in turn, was left to incubate for 15 min, while EFS was continued. Then, the selective 5-HT_4-receptor agonist, prucalopride was added to the organ bath solution under continued EFS and the response was followed for another 15 min.

6.3.3. Release study

The muscle strips (whole tissue or isolated CM strips) were transported in ice-chilled Krebs-Henseleit solution to the laboratory where the release study was conducted. Upon receipt, the Krebs-Henseleit solution was replaced by physiological salt solution (PSS; composition in mM: glucose 11.5, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgCl_2$ 1.2, KH_2PO_4 1.2, KCl 4.7, NaCl 112, choline 0.0015 and ascorbic acid 0.057). The human colon tissue preparations weighed 24.13 ± 2.81 (whole tissue CM; n = 34) and 7.32 ± 0.80 (isolated CM; n = 149) mg,

respectively. Whole tissue preparations used for release experiments were 1 cm in length and 3 mm in width, while measures of isolated CM preparations were more variable: 3-6 mm in length and 2-3 mm in width. A first series of experiments with single isolated CM strips revealed that the amount of total radioactivity (TR) released was too small to allow separation of the different components by HPLC. Therefore, a second series of experiments was performed whereby four isolated CM strips were mounted in one organ bath. As no differences between the different regions of the human colon were observed, the results were pooled. All human strips were used within 24 hours after surgery. Strips were mounted vertically without load between 2 platinum wire electrodes (40 x 0.5 mm) in 2 ml organ baths containing PSS, maintained at 37°C and gassed with carbogen (95% O_2 / 5% CO_2). Guanethidine ($4x10^{-6}$ M) was present in the medium throughout all experiments to avoid noradrenergic influences. EFS was applied by means of a Grass stimulator (S88, USA).

6.3.3.a. Experimental protocol of release study

Basically, the same method was used as described for the labelling of acetylcholine pools in pig gastric fundus (Leclere & Lefebvre, 2001). Briefly, using a peristaltic pump (Gilson Minipuls, France), the tissues were superfused at a rate of 2 ml/min during 60 min, and continuous EFS (40 V, 1 ms, 0.5 Hz) was applied the last 20 min. After this equilibration period, superfusion was stopped and the preparations were incubated for 30 min with [^{3}H]-choline (5 µCi/ml) during which the tissues were stimulated electrically (40 V, 1 ms, 2 Hz) in order to label their cholinergic transmitter stores.

After the labelling procedure, the strips were superfused (2 ml/min) for 60 min with PSS to remove loosely bound radioactivity. From now on the PSS contained hemicholinium-3 (10^{-5} M) to prevent the re-uptake of choline, physostigmine (10^{-5} M) to prevent the hydrolysis of acetylcholine and atropine (10^{-6} M) to prevent the auto-inhibition of acetylcholine release.

After the washout period, the strips were no longer superfused but the content of the organ bath, filled with 1 ml, was collected at 3 min intervals. A total of 35 samples was collected. 0.5 ml of the samples was mixed with 2 ml of the scintillator containing solution Ultima Gold (Canberra Packard, USA). The strips were stimulated twice for 2 min (S_1 and S_2; 15 V, 1 ms, 4 Hz), at 13 min (S_1, 5th sample), and 73 min (S_2, 25th sample) after the end of the washout period. Tetrodotoxin (TTX), calcium free medium and ω-conotoxin-GVIA were added 30 min (15th sample) before S_2, GR113808 was added 36 min (13th sample) before S_2

and prucalopride was added 15 min (20th sample) before S_2, and they remained present until the end of the experiment. At the end of the experiment, tissues were blotted and weighed.

6.3.3.b. Measurement of radioactivity and separation by HPLC of radioactive compounds

Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Canberra Packard, USA). External standardization was used to correct for counting efficiency. Electrical stimulation induced an increase in tritium overflow, not only in samples 5 (S_1) and 25 (S_2) but also in the two till four following samples. The stimulation-induced increase in tritium overflow was calculated by subtracting basal tritium overflow. Basal tritium overflow during the period of enhanced tritium overflow was calculated by fitting a regression line through the values of the three samples just before stimulation and the values of the three samples starting from where overflow had returned to basal values after stimulation.

The amount of [^{3}H]-acetylcholine, [^{3}H]-choline and [^{3}H]-phosphorylcholine in the samples was analysed by reverse phase HPLC (Bischoff Chromatography, Germany; Hyperchrome-HPLC-column, 250 x 4.6 mm, prepacked with HYPERSIL – ODS 5.0 µm). A 0.1 M phosphate buffer (pH 4.7) was used, containing methanol (8 vol %) and tetramethylammonium ($2x10^{-4}$ M). The flow was 0.5 ml/min and the effluent was collected in one min fractions. This is a suitable method to separate the different components as demonstrated previously (Leclere & Lefebvre, 2001).

HPLC was performed on one sample before S_1 and S_2 (sample 3 and sample 23, respectively), and on the sample during stimulation (sample 5 and 25). 100 µl of the sample was injected into the HPLC; 27 fractions were collected, and each fraction was mixed with 2 ml of Ultima Gold. Fractions 7 to 12 contained the peaks of [^{3}H]-phosphorylcholine and [^{3}H]-choline and were taken together to calculate the amount of [^{3}H]-phosphorylcholine and [^{3}H]-choline. Fractions 14 to 25 were summed to calculate the amount of [^{3}H]-acetylcholine (see Figure 6.2B and C). The absolute amount of [^{3}H]-phosphorylcholine plus [^{3}H]-choline and of [^{3}H]-acetylcholine was calculated by subtracting the background counting. Background counting was calculated by fitting a regression line through the values of the first 5 fractions and fractions 26 and 27. Finally, the percentage of [^{3}H]-acetylcholine in each sample was calculated.

6.3.4. Data analysis

6.3.4.a. Contractility study

For each individual (whole) muscle strip, the average contraction to five EFS pulse trains before addition of treatment or solvent was taken as 100% (called the initial value) and all contractions of this strip were related to this initial value. Repeated measures over time were analysed using PROC MIXED (SAS v PC 6.12) for data with an unbalanced covariance structure.

6.3.4.b. Release study

The ratios S_2/S_1 for TR and for tritiated acetylcholine were calculated. Results were compared by a paired or unpaired *t*-test, where appropriate. In the case where more than two responses were assessed, ANOVA was performed, followed by a *post-hoc t*-test corrected for multiple comparisons (Bonferroni procedure).

For both types of study, experimental data are expressed as means ± s.e.mean., *n* referring to the number of tissues obtained from different human specimens. *P* values of less than 0.05 were considered significant.

6.3.5. Drugs used

The following compounds were used in the contractility study (with their pharmaceutical names and respective suppliers given in parentheses): [1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate (GR113808), 4-amino-5-chloro-2,3-dihydro-N-(1-[3-methoxypropyl]-4-piperidinyl)-7-benzofurancarboxamide HCl (prucalopride; R093877) (Janssen Research Foundation, Belgium), atropine sulphate, carbachol, N^G-nitro-L-arginine (Janssen Chimica, Belgium), TTX (Serva, Germany). All compounds were dissolved in 0.9% NaCl solution, except for GR113808,

which was dissolved in 0.9% NaCl acidified with tartaric acid in the stock solution. Solutions were prepared freshly on the day of the experiment and all dilutions were made using 0.9% NaCl solution. The solvents did not affect EFS-induced contractions.

In the release study, L-ascorbic acid, atropine sulphate, choline chloride and guanethidine sulphate were obtained from Sigma (St. Louis, USA), hemicholinium-3-bromide from RBI (Natick, USA), methanol from Lab-Scan (Dublin, Ireland), [methyl-3H]-choline chloride (2775 GBq/mmol) from NEN (Boston, USA), physostigmine salicylate from Federa (Brussels, Belgium), prucalopride, and GR113808 from Janssen Research Foundation (Beerse, Belgium), tetramethylammonium chloride from Merck-Schuchardt (Hohenbrunn, Germany), and TTX and ω-conotoxin-GVIA from Alomone Labs (Jerusalem, Israel). The calcium free medium was prepared by replacing 2.5 mM $CaCl_2$ by 2.5 mM $MgCl_2$ in the PSS. Drugs were dissolved and diluted with deionized water. Stock solutions of TTX (10^{-3} M), prucalopride (10^{-2} M) and GR113808 (10^{-2} M) were kept frozen at –20°C. Dilutions were made on the day of the experiment.

6.4. Results

6.4.1. Contractility study

The human large intestinal (whole tissue) circular muscle strips were spontaneously active after they had been mounted in the organ baths. After wash-out of the contraction to carbachol (10^{-5} M), that induced a stable contraction in all preparations, the strips returned to baseline, and spontaneous activity was reduced. Spontaneous contractility was further reduced during EFS (to constitute roughly 5% of maximal contraction to EFS).

TTX ($3x10^{-7}$ M) and atropine (10^{-6} M) blocked the contractions elicited by EFS in every preparation. The effect of prucalopride ($3x10^{-7}$ M) on EFS-evoked contractions of human large intestinal preparations was variable. Prucalopride stimulated EFS-induced contractions in one specimen (by 26%), inhibited them in four out of eight preparations (inhibition ranging from 22-100%), and was without effect in three preparations (Figure 6.1A). On average, prucalopride ($3x10^{-7}$ M) tended to inhibit EFS-induced contractions, albeit this effect was not significant (71 ± 15% *versus* initial value, n = 8, $P > 0.05$). In all human muscle strips, GR113808 (10^{-7} M) prevented any effect of prucalopride (111 ± 8 % *versus* initial value, n = 8, $P > 0.05$, Figure 6.1B).

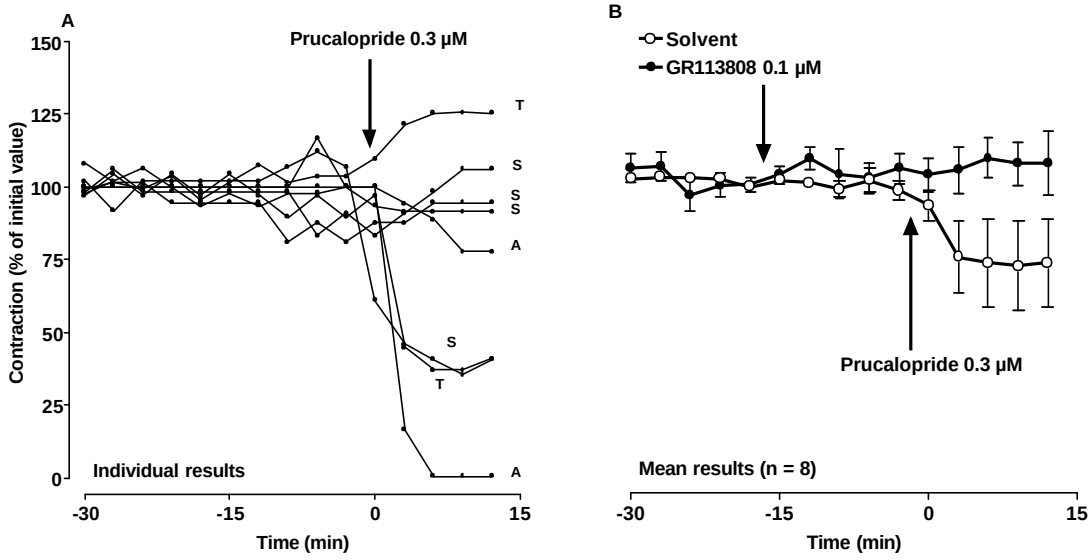

Figure 6.1 Effect of prucalopride on EFS-evoked contractions of human large intestine circular muscle. All experiments were carried out in the presence of L-NNA (10^{-4} M). The left panel shows individual data points of the effect of prucalopride, that are connected by a within-specimen solid line. The origin of the tissues in the large intestine is indicated : A, ascending colon, T, transverse colon, S, sigmoid colon. The right panel shows mean contraction ± s.e.mean. (*n* = 8), calculated as percentage of the mean contraction to five EFS pulse trains immediately prior to any addition of compound (initial value). The response to prucalopride after addition of solvent or GR113808 is shown.

6.4.2. Release study

6.4.2.a. Whole tissue of human colon

In human tissues, field stimulation caused a clear-cut increase in total radioactivity (TR) and 9-12 min were required after stimulation to re-establish the basal release of tritium (Figure 6.2A). The mean amount of TR in samples 3 and 23 (pre stimulation), and in the samples with increased release due to stimulation (S_1 and S_2), is given in Table 6.1. This yielded a S_2/S_1 ratio for TR of 0.67 ± 0.02 (*n* = 8). [^{3}H]-Acetylcholine could be detected when HPLC was used to separate the different components present in the samples. In basal conditions, TR contained more [^{3}H]-phosphorylcholine plus [^{3}H]-choline then [^{3}H]-acetylcholine. Stimulation induced a pronounced increase in the release of [^{3}H]-acetylcholine, although there was also a two- to threefold increase in the release of [^{3}H]-phosphorylcholine and [^{3}H]-choline during stimulation (Figure 6.2B and C). The mean amounts of [^{3}H]-choline plus [^{3}H]-phosphorylcholine, and [^{3}H]-acetylcholine before and during stimulation, as well as the percentage of [^{3}H]-acetylcholine released are given in Table 6.1. The S_2/S_1 ratio for [^{3}H]-acetylcholine was 0.71 ± 0.06 (*n* = 7).

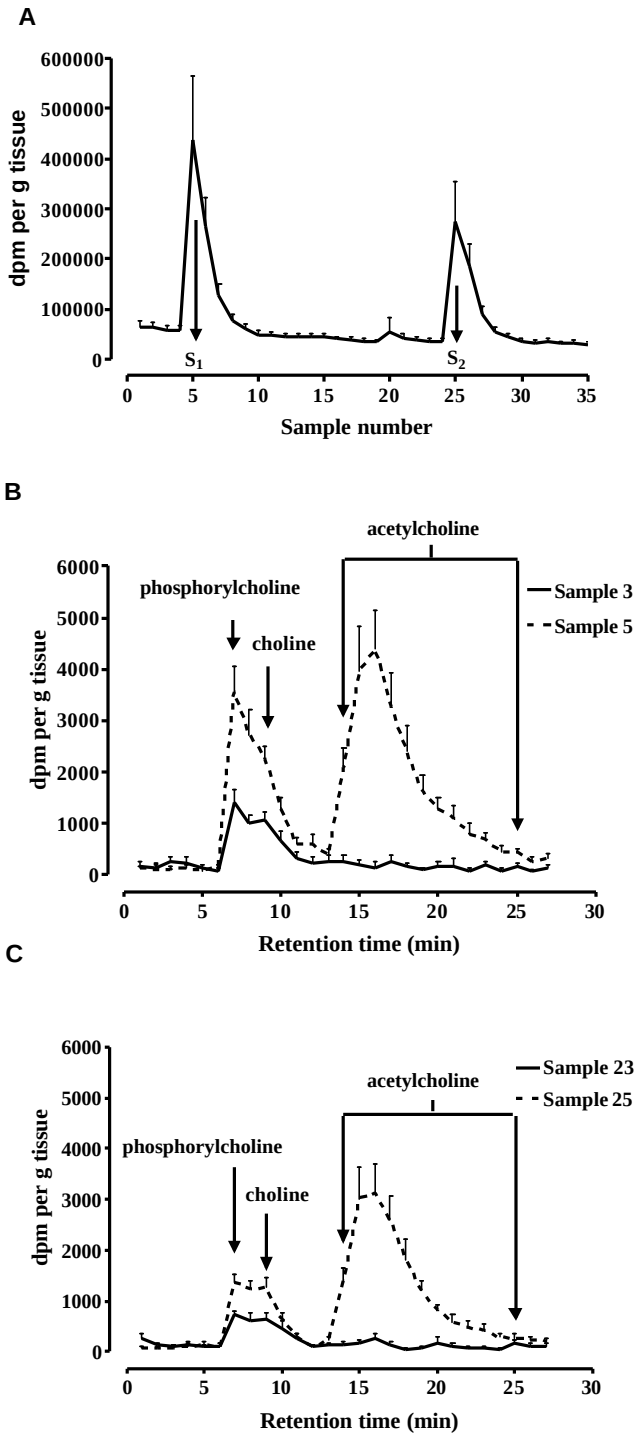

Figure 6.2 Influence of electrical field stimulation on the release of total radioactivity (TR) and [^{3}H]-acetylcholine from control whole tissue strips of human colon. (A) TR outflow from human colon whole tissue preparations pre-incubated with [^{3}H]-choline. The abscissa starts at the end of the washout period. Tissues were stimulated twice (S_1 and S_2: 15 V, 1 ms, 4 Hz, 2 min), and the superfusate (1 ml) was collected in 3 min fractions. (B) HPLC-separation of the radioactive outflow before (sample 3) and during (sample 5) S_1. Arrows indicate the peaks for [^{3}H]-phosphorylcholine, [^{3}H]-choline and [^{3}H]-acetylcholine. (C) HPLC-separation of the radioactive outflow before (sample 23) and during (sample 25) S_2. 100 µl of the samples were injected into the HPLC, and every min 500 µl was collected. Results are given as mean ± s.e.mean (panel A, n = 8; panels B and C, n = 7).

Table 6.1 Outflow of total radioactivity, [^{3}H]-phosphorylcholine plus [^{3}H]-choline and [^{3}H]-acetylcholine for control whole tissue (WT) and isolated (ISO) circular muscle strips of human colon (4 isolated circular muscle strips were mounted in one organ bath for each experiment).

		TR	Ph.-Ch. + Ch.	ACh	% ACh
WT	Sample 3	58 280 ± 9 830	37 440 ± 4 190	6 180 ± 4 400	9 ± 5
	S_1	698 840 ± 196 510	100 180 ± 14 400***	178 360 ± 33 250***	63 ± 4***
	Sample 23	35 760 ± 7 140	19 910 ± 4 600	3 690 ± 3 020	7 ± 4
	S_2	466 090 ± 132 530	41 180 ± 4 050$^{\#\#}$	125 370 ± 23 260$^{\#\#\#}$	72 ± 4$^{\#\#\#}$
	S_2/S_1	**0.67 ± 0.02**		**0.71 ± 0.06**	
	n	8	7	7	7
ISO	Sample 3	75 200 ± 7 640	69 060 ± 12 480	2 920 ± 1 590	5 ± 2
	S_1	850 950 ± 181 690	232 520 ± 69 680*	32 086 ± 57 690**	59 ± 5***
	Sample 23	48 040 ± 6 930	41 480 ± 6 280	3 850 ± 2 060	7 ± 3
	S_2	509 340 ± 95 460	89 330 ± 21 460$^{\#}$	202 760 ± 38 710$^{\#\#}$	68 ± 5$^{\#\#\#}$
	S_2/S_1	**0.63 ± 0.03**		**0.63 ± 0.03**	
	n	6	6	6	6

S_1 and S_2 for total radioactivity (TR) are the sum of respectively sample 5 and sample 25 and the following samples (see text), while S_1 and S_2 for [^{3}H]-phosphorylcholine plus [^{3}H]-choline (Ph.-Ch. + Ch.) and [^{3}H]-acetylcholine (ACh) are respectively samples 5 and 25. Radioactivity is expressed as dpm per g tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: Significantly different from values before S_1 (sample 3). $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$: Significantly different from values before S_2 (sample 23). Results are given as mean ± s.e.mean.

TTX ($3x10^{-6}$ M; $n = 5$), ω-conotoxin-GVIA (10^{-6} M; $n = 1$) or removal of extracellular calcium ($n = 2$) did not influence basal release of TR. TTX nearly abolished the electrically-evoked tritium release as compared with control. In control tissues ($n = 5$), the S_2/S_1 ratio for release of TR was 0.70 ± 0.02. The S_2/S_1 ratio for TR after superfusion with TTX was 0.08 ± 0.01 ($P < 0.001$). The S_2/S_1 ratio for TR in the presence of ω-conotoxin was 0.16 ($n = 1$), while the ratio was 0.67 for control tissue. In the absence of calcium ($n = 2$), the S_2/S_1 ratio for TR was 0.05 and 0.13 , while this was 0.65 and 0.67 in control tissues.

Prucalopride ($3x10^{-7}$ M) did not alter the basal efflux of TR. However, prucalopride increased the electrically evoked release of TR and [^{3}H]-acetylcholine; the mean increase in electrically evoked release of [^{3}H]-acetylcholine by prucalopride was significant (Figure 6.3A). The 5-HT$_4$-receptor antagonist GR113808 (10^{-7} M) did not alter the basal release of TR ($n = 8$), nor did it influence the S_2/S_1 ratio for TR (S_2/S_1 ratio for control: 0.69 ± 0.05, $n = 4$, and for GR113808: 0.66 ± 0.03, $n = 4$, $P > 0.05$) and for [^{3}H]-acetylcholine (S_2/S_1 ratio for control: 0.75 ± 0.01, $n = 3$, and for GR113808: 0.78 ± 0.05, $n = 3$, $P > 0.05$). When added before prucalopride, GR113808 prevented the increase of the electrically evoked release of TR and reduced that of [^{3}H]-acetylcholine ($n = 4$) by prucalopride (Figure 6.3A).

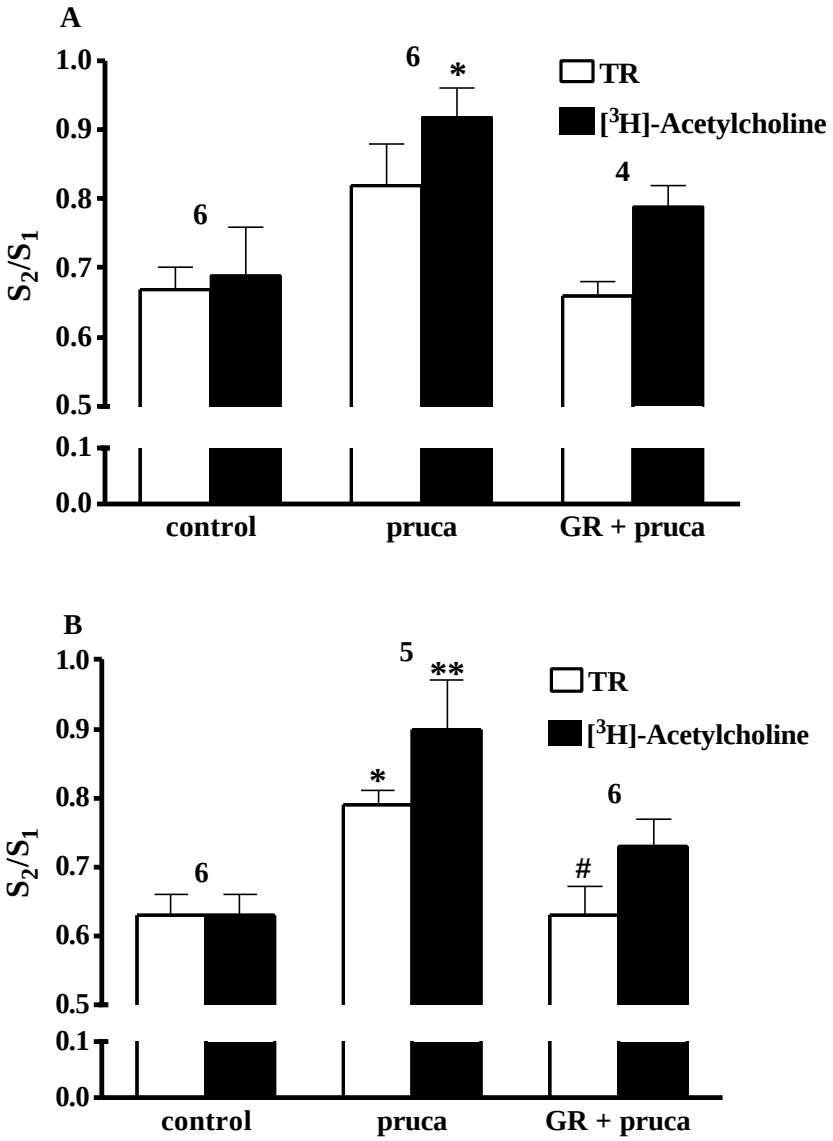

Figure 6.3 Effects of prucalopride (pruca) and prucalopride in the presence of GR113808 (GR) on the electrically evoked release of total radioactivity (TR) and [^{3}H]-acetylcholine from preparations of (A) human colon whole tissue preparations and (B) human colon isolated circular muscle preparations pre-incubated with [^{3}H]-choline (in B, 4 isolated circular muscle strips were mounted together in one organ bath for each experiment). Tissues were stimulated twice (S_1 and S_2: 15 V, 1 ms, 4 Hz, 2 min); GR113808 (10^{-7} M) was added 36 min and prucalopride ($3x10^{-7}$ M) 15 min before S_2. The electrically evoked efflux by S_2 is expressed as a ratio of that by S_1. Each column represents the mean ± s.e.mean. *, ** $P < 0.05$, 0.01: Significantly different from control. # $P < 0.05$: Significantly different from pruca. Numbers above the columns refer to the number of experiments.

6.4.2.b. Isolated circular smooth muscle of human colon

Electrical stimulation induced an increase in tritium overflow in isolated human colon CM, and 6-12 min were required after stimulation to re-establish the basal release of tritium. The S_2/S_1 ratio for TR was 0.53 ± 0.03 (n = 11). However, the amount of TR released by the

very small isolated CM strips was very low, and reached the detection limit of the HPLC; a reliable separation by HPLC of the different components present in the samples was therefore not feasible.

TTX ($3x10^{-6}$ M; n = 4), ω-conotoxin-GVIA (10^{-6} M; n = 1) or removal of extracellular calcium (n = 2) did not influence basal release of TR. TTX nearly abolished the electrically-evoked tritium release as compared with control in three out of four experiments (S_2/S_1 ratios for release of TR: control, 0.55 ± 0.03 *versus* TTX, 0.13 ± 0.03; n = 3; $P < 0.001$). In one experiment, the electrically evoked tritium release was not abolished by TTX (S_2/S_1 ratios for release of TR: control, 0.53 *versus* TTX, 0.51), but in the whole tissue preparation of the same patient, TTX abolished the electrically-evoked tritium release (S_2/S_1 ratios for release of TR: control, 0.65 *versus* TTX, 0.09). The S_2/S_1 ratio for TR in the presence of ω-conotoxin was 0.15 (n = 1), while the ratio was 0.49 for control tissue. After superfusion with calcium free medium (n = 2), the S_2/S_1 ratio for TR was 0.24 and 0.10, while this was 0.59 and 0.49 in control tissues.

Prucalopride ($3x10^{-7}$ M) did not alter the basal efflux of TR. However, prucalopride significantly enhanced the amount of TR (S_2/S_1 ratio for TR: control, 0.54 ± 0.03 *versus* prucalopride, 0.77 ± 0.05; $P < 0.001$; n = 9). GR113808 (10^{-7} M) did not alter the basal release of TR (n = 7). When added before prucalopride, GR113808 antagonised the increase of the electrically evoked release of TR by prucalopride (S_2/S_1 ratio for TR: 0.62 ± 0.02; n = 7).

In order to be able to measure [^{3}H]-acetylcholine by HPLC, an additional series of experiments was performed where four isolated CM strips were mounted together in one organ bath. This allowed to separate the different components by HPLC, as enough radioactivity was released.

In basal conditions, released radioactivity contained only a small fraction of [^{3}H]-acetylcholine. Stimulation induced a significant increase in the release of [^{3}H]-acetylcholine, although there was some increase in the release of [^{3}H]-phosphorylcholine and [^{3}H]-choline during stimulation. The mean amounts of TR, [^{3}H]-choline plus [^{3}H]-phosphorylcholine and [^{3}H]-acetylcholine before and during stimulation, as well as the percentage of [^{3}H]-acetylcholine released are given in Table 6.1. The S_2/S_1 ratios were 0.63 ± 0.03 for both TR and [^{3}H]-acetylcholine (n = 6).

TTX ($3x10^{-6}$ M; n = 5) did not influence basal release of TR. However, TTX nearly abolished the electrically-evoked tritium release as compared with control (the S_2/S_1 ratios for

release of TR and [^{3}H]-acetylcholine for control tissues were respectively 0.58 ± 0.03 and 0.69 ± 0.06, while the ratios for TTX were respectively 0.15 ± 0.03 and 0.03 ± 0.01; $P < 0.001$; $n = 5$).

Prucalopride ($3x10^{-7}$ M) did not alter the basal efflux of TR. However, prucalopride significantly enhanced the amount of TR and [^{3}H]-acetylcholine ($P < 0.01$; $n = 5$; Figure 6.3B). GR113808 (10^{-7} M; $n = 7$), added before prucalopride, had no effect on basal release, however, it completely antagonised the increase by prucalopride of the electrically evoked release of TR (Figure 6.3B). It also reduced the increase by prucalopride of the electrically evoked release of [^{3}H]-acetylcholine; although the S_2/S_1 ratio for [^{3}H]-acetylcholine in the presence of GR113808 and prucalopride was not significantly different from that in the presence of prucalopride alone, it was also not significantly different from the S_2/S_1 ratio in control tissues.

6.5. Discussion

The data presented here suggest that the human colon circular muscle is endowed with 5-HT$_4$-receptors located on cholinergic nerves, mediating facilitation of cholinergic neurotransmission resulting in enhanced contraction. Colonic circular muscle may be the first single muscle layer expressing both an inhibitory smooth muscle and an excitatory neuronal 5-HT$_4$-receptor component.

Stimulation-induced contractions and release of TR and [^{3}H]-acetylcholine in colon tissues were reduced by TTX and calcium free medium (the latter only tested in the release study). This indicates a neuronal release dependent upon the opening of sodium channels, and the presence of calcium in the external medium, respectively. The blockade of EFS-induced contractions by atropine in all whole tissue muscle strips, suggested that these nerves are cholinergic in origin. Calcium enters the cholinergic nerves *via* N-type calcium channels as ω-conotoxin-GVIA abolished the electrically evoked release of TR. [^{3}H]-Choline and [^{3}H]-phosphorylcholine may be released by leakage from the nerve terminals in basal conditions; they indeed formed the major part of basally released radioactivity, and this was not influenced in the presence of TTX and ω-conotoxin. TTX abolished stimulation-induced increase of [^{3}H]-acetylcholine, while stimulation-induced increase of TR was clearly reduced but not abolished. This supports the full neuronal release of acetylcholine, but suggests that stimulation induces some non-neuronal release of [^{3}H]-choline and [^{3}H]-phosphorylcholine.

Prucalopride has been shown to be a selective 5-HT_4-receptor agonist in isolated tissues of rodents (Briejer *et al.*, 2001a), dogs (Prins *et al.*, 1999) as well as humans (Prins *et al.*, 2000b). At $3x10^{-7}$ M, prucalopride stimulates 5-HT_4-receptors on cholinergic nerves in canine ascending and descending colon and human large intestine longitudinal muscle (Prins *et al.*, 2000a). Here, this concentration of prucalopride enhanced contractions due to EFS in some human colonic muscle strips, an effect that was absent in the presence of the selective 5-HT_4-receptor antagonist GR113808 (10^{-7} M) (Gale *et al.*, 1994). The competitive nature of the 5-HT_4-receptor antagonism produced by GR113808 has been confirmed in studies with canine and human gastrointestinal preparations (pK_B 9.1 (Prins *et al.*, 1999); pK_B 9.4 (Prins *et al.*, 2000b)), showing that 10^{-7} M would induce over a 2-log unit shift of the curve to a 5-HT_4-receptor agonist. Prucalopride at $3x10^{-7}$ M induces an approximate EC_{50}-EC_{90} response *via* 5-HT_4-receptors as determined in various bioassays of rodent (Briejer *et al.*, 2001a), dog (Prins *et al.*, 1999) and human gastrointestinal tissue (Prins *et al.*, 2000b). Hence, GR113808 (10^{-7} M) is expected to block a 5-HT_4-receptor-mediated response to prucalopride ($3x10^{-7}$ M). In this manner, it is conceivable that the enhancement of EFS-induced circular muscle contraction after prucalopride may be due to activation of 5-HT_4-receptors triggering a cholinergic pathway. This was strongly corroborated by the prucalopride-evoked systematic increase in TR and [^{3}H]-acetylcholine efflux from whole tissue CM, an effect that was prevented by GR113808. Still, the whole tissue CM strips contain a small amount of longitudinally directed muscle, where 5-HT_4-receptors are present on the cholinergic nerves (Prins *et al.*, 2000a) and that might contribute to the acetylcholine release detected in the whole tissue preparations. However, also in isolated CM strips, only containing circular muscle and nerve endings between the muscle fibers, prucalopride stimulated TR and [^{3}H]-acetylcholine release and this effect was antagonised by GR113808, although to a smaller extent in the case of [^{3}H]-acetylcholine. It is unlikely that prucalopride acts on another (5-HT-)receptor as it possesses high selectivity for 5-HT_4-receptors and was not used in a supramaximal concentration (Briejer *et al.*, 2001a). Hence, in human colon CM, excitatory 5-HT_4-receptors are presumably located on cholinergic nerves within the circular muscle wall. As GR113808 did not influence the S_2/S_1 ratio for TR and [^{3}H]-acetylcholine, it is unlikely that electrical stimulation causes the release of endogenous 5-HT which might act on 5-HT_4-receptors to enhance [^{3}H]-acetylcholine release.

The question can then be raised why the corresponding functional response, i.e. increased contraction after cholinergic nerve stimulation in the presence of the 5-HT_4-receptor agonist, is not systematically observed in the contractility study as prucalopride even reduced

the EFS-induced contractions in some strips. This is explained by the presence of inhibitory smooth muscle 5-HT_4-receptors in human colonic circular muscle, for which a number of reports have provided compelling evidence (Prins *et al.*, 2000b, Meulemans *et al.*, 1995, Tam *et al.*, 1994, McLean *et al.*, 1996). Inducing contractions by EFS does not prevent prucalopride from activating these smooth muscle receptors, irrespective of prucalopride stimulating neuronal receptors as well. Hence, functional antagonism of the EFS-induced contraction is expected due to activation of the inhibitory smooth muscle 5-HT_4-receptors. In view of the above, across-specimen variation in the expression of both functional responses (inhibition *versus* stimulation of contractility) may explain the heterogenous nature of the prucalopride-induced response in the contractility study. It is unlikely that the region determines the EFS-induced response to prucalopride, as in one specimen of transverse colon, EFS-induced contractions were enhanced by prucalopride, while in another specimen these contractions were inhibited. Nevertheless, the outcome of this study is ultimately novel, as the colon circular muscle may represent the very first tissue entity in which both locations and functions of 5-HT_4-receptors have been detected.

The mechanisms underlying the triggering and propulsion of giant contractions over longer distances of large intestine are as yet unknown, but the outcome of the current study may provide the first evidence to explain the prucalopride-induced occurrence of GMCs in conscious dogs (Briejer *et al.*, 2001b). The blockade of colonic motility and GMCs by the non-selective muscarinic cholinoceptor antagonist atropine and the selective M_3 cholinoceptor antagonist zamifenacin (Prins *et al.*, 2001) indicates that these phenomena are highly dependent on the presence of cholinergic tone mediated *via* mainly M_3 cholinoceptors. It is thus conceivable that the outflow of acetylcholine from cholinergic neurones that fire in a GMC, is enhanced in the presence of a selective 5-HT_4-receptor agonist. In cases where cholinergic drive underlying GMCs may be low (in some forms of constipation (Sarna *et al.*, 2000)), or not yet maximal (normal subjects), one may expect higher GMC frequency and amplitude after 5-HT_4-receptor activation. Possibly the well-co-ordinated interplay between constant muscle activity inhibition *via* smooth muscle 5-HT_4-receptor activation, alternated by short-lasting high-amplitude contractions due to activated 5-HT_4-receptors on excitatory cholinergic nerves facilitates propulsion of large bowel contents. These combined smooth muscle inhibitory and cholinergic facilitatory activities may be the main mechanism by which 5-HT_4-receptor agonists are potent and efficacious stimulators of co-ordinated colonic motility and stool frequency.

In conclusion, this study illustrates the presence of 5-HT_4 receptors on cholinergic nerves within the circular muscle of the human colon.

6.6. References

BOURAS, E.P., CAMILLERI, M., BURTON, D.D. & MCKINZIE, S. (1999). Selective stimulation of colonic transit by the benzofuran $5HT_4$ agonist, prucalopride, in healthy humans. *Gut*, **44**, 682 - 686

BRIEJER, M.R., BOSMANS, J.-P., VAN DAELE, P., JURZAK, M., HEYLEN, L., LEYSEN, J.E., PRINS, N.H. & SCHUURKES, J.A.J. (2001a). The *in vitro* pharmacological profile of prucalopride, a novel enterokinetic compound. *Eur. J. Pharmacol.*, **423**, 71 - 83

BRIEJER, M.R., PRINS, N.H. & SCHUURKES, J.A.J. (2001b). Effects of the enterokinetic prucalopride (R093877) on colonic motility in fasted dogs. *Neurogastroenterol. Mot.*, **13**, 465 - 472

EMMANUEL, A.V., KAMM, M.A., ROY, A.J. & ANTONELLI, K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut*, **42**, 511 - 516

GALE, J.D., GROSSMAN, C.J., WHITEHEAD, JW., OXFORD, A.W., BUNCE, K.T. & HUMPHREY, P.P. (1994). GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. *Br. J. Pharmacol.*, **111**, 332 - 338

HEDGE, S.S. & EGLEN, R.M. (1996). Peripheral 5-HT_4 receptors. *FASEB*, **10**, 1398 - 1407

KARAUS, M. & SARNA, S.K. (1987). Giant migrating contractions during defecation in the dog colon. *Gastroenterology*, **92**, 925 - 933

LECLERE, P.G. & LEFEBVRE, R.A. (2001). Influence of nitric oxide donors and of the α_2-agonist UK-14,304 on acetylcholine release in the pig gastric fundus. *Neuropharmacology*, **40**, 270 - 278

MCLEAN, P.G. & COUPAR, I.M. (1996). Stimulation of cyclic AMP formation in the circular smooth muscle of human colon by activation of 5-HT_4-like receptors. *Br. J. Pharmacol.*, **117**, 238 - 239

MEULEMANS, A.L., GHOOS, E., CHEYNS, P. & SCHUURKES, J.A.J. (1995). 5-HT-induced relaxations of human sigmoid colon are mediated *via* 5-HT_4 receptors. *Pflüger's Arch. Eur. J. Physiol.*, **429**, R9

PRINS, N.H., AKKERMANS, L.M.A., LEFEBVRE, R.A. & SCHUURKES, J.A.J. (2000a). 5-HT_4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br. J. Pharmacol.*, **131**, 927 - 932

PRINS, N.H., SHANKLEY, N.P., WELSH, N.J., BRIEJER, M.R., LEFEBVRE, R.A., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (2000b). An improved *in vitro* bioassay for the study of 5-HT_4 receptors in the human isolated large intestinal circular muscle. *Br. J. Pharmacol.*, **129**, 1601 - 1608

PRINS, N.H., VAN HASELEN, J.F.W.R., LEFEBVRE, R.A., BRIEJER, M.R., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (1999). Pharmacological characterization of 5-HT_4 receptors mediating relaxation of canine isolated rectum circular smooth muscle. *Br. J. Pharmacol.*, **127**, 1431 - 1437

PRINS, N.H., VER DONCK, L., EELEN, J., GHOOS, E. & SCHUURKES, J.A.J. (2001). M_3 cholinoceptor blockade inhibits dog colonic motility and antagonizes 5-HT_4 receptor agonist-induced giant migrating contractions. *Gastroenterology*, **120** (Suppl.), 4047

SARNA, S.K., BRIEJER, M.R. & SCHUURKES, J.A.J. (2000). 5-HT_3/5-HT_4 receptors in motility disorders – New therapeutic agents. In : *Drug Development : Molecular Targets for Gastrointestinal Diseases.* Eds. Gaginella, T.S. & Guglietta, A. pp. 177 - 202

TAM, F.S., BUNCE, K.T., HILLIER, K. & GROSSMAN, C. (1994). Characterization of the 5-hydroxytryptamine receptor type involved in inhibition of spontaneous activity of human isolated colonic circular muscle. *Br. J. Pharmacol.*, **113**, 143 - 150

CHAPTER 7

GENERAL DISCUSSION

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Cholinergic neurones are important in the regulation of GI motility. The released acetylcholine acts on muscular muscarinic receptors, causing contraction of the muscle. Other neurotransmitters can interfere with acetylcholine at the postsynaptic level whereby the cholinergic contraction will be modulated, and/or presynaptically whereby the release of acetylcholine will be enhanced or inhibited. The purpose of this thesis was to investigate the modulation of cholinergic neurotransmission at the post- and presynaptic level in stomach and colon. Contraction and release experiments were performed. Even when the contraction is induced by electrical stimulation of the cholinergic neurones and release of acetylcholine, a change in contraction does not necessarily reflect a change in acetylcholine release. In release experiments, tissues are incubated with [^{3}H]-choline in order to label the vesicles with [^{3}H]-acetylcholine, that will be released upon electrical stimulation. The radioactivity released can be measured and HPLC of the samples allows to determine the amount of [^{3}H]-acetylcholine. The presynaptic effect of substances can then be assessed directly. Experiments were performed on whole tissue strips, cut in the direction of the circular muscle, from pig and human gastric fundus, and human colon. For human colon, also strips that only contained circular muscle were studied.

7.1. Interaction of the nitrergic and cholinergic innervation of the gastric fundus

The study focused on the interaction between acetylcholine and NO, as the latter is the principle NANC inhibitory neurotransmitter. During the course of the contraction experiments, the basal tone of the circular muscle strips of pig gastric fundus tended to increase, a phenomenon also described by other authors (Mandrek and Kreis, 1992; Lefebvre *et al.*, 1995). During the experiments to characterise the muscarinic receptors, it was shown that indomethacin, a cyclo-oxygenase inhibitor preventing the synthesis of prostanoids, prevented the increase in basal tone, suggesting that the progressive synthesis of prostanoids is responsible for the increase in basal tone. The acetylcholinesterase inhibitor physostigmine induced contraction in pig gastric fundus, suggesting that there is some acetylcholine release under basal conditions. When the different components in samples released under basal

conditions were separated by HPLC in the release experiments, small amounts of [^{3}H]-acetylcholine were detected, confirming that some acetylcholine is released under basal conditions. The release is probably due to leakage out of the cholinergic neurones and not to action potential propagation as tetrodotoxin did not influence basal [^{3}H]-acetylcholine release of the strips. The acetylcholine released apparently does not contribute to the basal tone as atropine did not lower the tone of the tissues. NO is also released under basal conditions as the NOS inhibitor L-NAME induced contraction. Tetrodotoxin did not influence basal tone suggesting that NO is released from the nitrergic neurones without action potential propagation.

Electrically-induced responses (relaxations or contractions) in pig gastric fundus strips were blocked by tetrodotoxin, illustrating the neuronal origin of the responses, except for contractions at higher frequencies of stimulation. These remaining contractions are probably due to direct smooth muscle cell activation. The nicotinic receptor blocker hexamethonium did not affect the responses to electrical stimulation, indicating that postganglionic neurones are activated. Electrical stimulation of pig gastric fundus strips induced relaxations at low frequencies, and contractions at higher frequencies. The NOS inhibitor L-NAME and the acetylcholinesterase inhibitor physostigmine reversed the electrically-induced relaxations into contractions while they enhanced the contractions; the muscarinic receptor antagonist atropine blocked the contractions, and relaxations occurred at all frequencies in the absence of L-NAME. These results suggest that both NO and acetylcholine are released at all stimulation frequencies, but the nitrergic contribution is dominant at the lower while the cholinergic contribution is dominant at the higher stimulation frequencies, corresponding to what has been observed in the opossum lower oesophageal sphincter and human gastric fundus (Cellek & Moncada, 1997; Tonini *et al.*, 2000). When released together, NO and acetylcholine can certainly be expected to functionally antagonise each other at the postsynaptic level. However, in guinea-pig (Wiklund *et al.*, 1993; Kilbinger & Wolf, 1994; Hebeiβ & Kilbinger, 1996, 1998), canine (Hryhorenko *et al.*, 1994) and mouse (Mang *et al.*, 2000) ileum, as well as in rat trachea (Sekizawa *et al.*, 1993), it has been shown that NO is also able to modulate cholinergic responses by presynaptic inhibition of acetylcholine release. To study whether NO might interfere with acetylcholine release in pig gastric fundus, we investigated the influence of the NO donor SNP on contractions to either electrical stimulation causing the release of endogenous acetylcholine, or exogenously applied acetylcholine. This was done in the presence of L-NAME to exclude a possible inhibitory effect of endogenously released NO on acetylcholine release. If NO inhibits acetylcholine release, it can be expected that SNP

inhibits contractions to electrical stimulation more than those to exogenous acetylcholine. The effect of SNP on contractions induced by electrical stimulation or exogenous acetylcholine was always similar, suggesting that the interaction between NO and acetylcholine in the pig gastric fundus occurs only at the postsynaptic level. This corresponds with results obtained in guinea-pig gastric fundus (Milenov & Kalfin, 1996), opossum lower oesophageal sphincter (Cellek & Moncada, 1997), guinea-pig trachea (Brave *et al.*, 1991) and human airways (Ward *et al.*, 1993). However, it is not possible to determine with certainty the site(s) of action of NO (presynaptic inhibition of acetylcholine release *versus* postsynaptic functional antagonism of acetylcholine) from functional experiments. For this reason, the effect of NO on [^{3}H]-acetylcholine release from cholinergic nerve endings in the pig gastric fundus was studied. Neither L-NAME nor NO donors interfered with [^{3}H]-acetylcholine release. No evidence for presynaptic modulation of acetylcholine release by NO was thus obtained supporting our suggestion from the contraction experiments that NO and acetylcholine antagonise each other only at the postsynaptic level. In human gastric fundus, it had been shown that electrical stimulation induced the release of acetylcholine and NO, and functional antagonism between NO and acetylcholine was suggested on the basis of functional results (Tonini *et al.*, 2000). As it was our goal to compare the results obtained in pig and human gastric fundus, the effect of L-NAME and SNP on [^{3}H]-acetylcholine release was also measured in human gastric fundus. Neither L-NAME nor SNP influenced [^{3}H]-acetylcholine release, illustrating that also in human gastric fundus NO only functionally antagonises acetylcholine at the postsynaptic level.

7.2. Characterisation of pre- and postsynaptic muscarinic receptors

Stimulation-induced cholinergic contractions were blocked by atropine in the pig gastric fundus, indicating that the released acetylcholine activates muscular muscarinic receptors. Pharmacological studies in many tissues found evidence that M_3 receptors are involved in the contraction of smooth muscle cells. However, radioligand binding studies demonstrated a high predominance of M_2 receptors, whereby e.g. in human stomach 79% of the muscular muscarinic receptors belong to the M_2 subtype, and the remaining 21% are M_3 receptors (Bellido *et al.*, 1995). Although M_3 receptors mediate muscle contraction in the GI tract, a role for M_2 receptors, in terms of modulation of cyclic AMP driven relaxation, is evident under specialized experimental conditions. Indeed, when the M_3 receptors are

blocked, and guanylyl cyclase is activated, then acetylcholine can contract the tissues *via* stimulation of muscular M_2 receptors (see reviews Eglen *et al.*, 1996; Caulfield & Birdsall, 1998). To characterise pharmacologically the muscarinic receptor subtype, one needs to test a series of subtype-preferring muscarinic antagonists because of the lack of selective muscarinic receptor antagonists. By comparing the influence of these muscarinic receptor antagonists on contractions to exogenously applied acetylcholine, it is possible to characterise the postsynaptic muscarinic receptor subtype. The rank order of the potencies as well as the affinity constants of the muscarinic antagonists suggested that the postsynaptic muscarinic receptors in pig gastric fundus, responsible for the contraction of the circular muscle, belong to the M_3 subtype. This is in agreement with observations in other tissues (see e.g. Dietrich & Kilbinger, 1995; Shi & Sarna, 1997; Preiksaitis & Laurier, 1998). However, we did not find any evidence for the presence of postsynaptic M_2 receptors, although a binding study in pig gastric fundus suggested that two different muscular muscarinic receptors are present, one of them most likely belonging to the M_2 subtype (Herawi *et al.*, 1988).

In many tissues, acetylcholine interferes with its own release, mostly inhibiting the release of acetylcholine, although a facilitatory effect has also been observed (see reviews Stärke *et al.*, 1989; Grimm *et al.*, 1994). Atropine, added before the second stimulation in our release experiments, increased electrically-induced [^{3}H]-acetylcholine release in pig and human gastric fundus, suggesting the presence of inhibitory presynaptic muscarinic autoreceptors on cholinergic nerve endings innervating the circular muscle layer of pig and human gastric fundus. An attempt was made to characterise pharmacologically the inhibitory presynaptic muscarinic receptors on the cholinergic neurones of the pig gastric fundus by comparing the effect of a series of subtype-preferring muscarinic receptor antagonists on electrically-induced contractions and acetylcholine release; this was not done for human gastric fundus due to the limited number of stomachs available. AF-DX 116 and methoctramine, two M_2 subtype preferring muscarinic antagonists, increased the electrically-induced contractions at the lowest concentrations tested, and they were more potent in facilitating the evoked tritium release (a presynaptic effect) than in inhibiting the contractile response (a postsynaptic effect). These results suggest the presence of a presynaptic inhibitory M_2 receptor. However, the pIC_{50} values for the presynaptic effect of the antagonists on tritium release did not correlate with published pK_i values for the muscarinic antagonists at the M_2 receptor subtype, excluding that the presynaptic inhibitory muscarinic receptors belong to the M_2 subtype. A significant correlation was found between the presynaptic pIC_{50} values for the tested antagonists and the pK_i values for M_4 receptors from

literature, but results with the selective M_4 receptor antagonist MT-3 did not allow to conclude to the presence of presynaptic M_4 receptors. Indeed, MT-3 had no effect on electrically-induced contractions and tritium release in concentrations up to 10^{-8} M, where an effect of MT-3 can be expected as the pK_i value of MT-3 on M_4 receptors is between 8.9 - 8.3 (Adem & Karlsson, 1997; Olianas *et al.*, 1999; Jerusalinsky *et al.*, 2000). A similar and significant correlation was obtained between the presynaptic pIC_{50} values for the antagonists tested, and binding affinities at M_1, M_3 and M_5 receptors from literature, making it impossible to classify the presynaptic inhibitory muscarinic receptor as one of these muscarinic receptor subtypes. An explanation for these results might be the presence of more than one type of muscarinic receptor on the cholinergic nerve endings, inhibiting or even stimulating acetylcholine release. In the guinea-pig LMMP preparation, two types of presynaptic muscarinic receptors were identified, an inhibitory M_3 and a facilitatory M_1 receptor (Soejima *et al.*, 1993). In canine LMMP preparations, the presence of presynaptic M_3 receptors was demonstrated, but the presence of another presynaptic muscarinic subtype could not be excluded (Kostka *et al.*, 1989).

7.3 Presynaptic modulation of acetylcholine release in the gastric fundus

Throughout the GI tract, nitrergic neurones often contain VIP besides of nNOS (Furness *et al.*, 1992; Lefebvre *et al.*, 1995). This is also the case in human gastric fundus, where VIP desensitisation reduced L-NNA-resistant relaxations induced by electrical stimulation at higher frequencies, suggesting that VIP is released (Tonini *et al.*, 2000). In rabbit stomach, electrically-induced cholinergic contractions were inhibited by VIP (Baccari *et al.*, 1994); the authors suggested that VIP might inhibit the release of acetylcholine, although this type of contraction studies cannot exclude that VIP only antagonises acetylcholine at the postsynaptic level. In guinea-pig stomach, VIP inhibits electrically-induced [^{3}H]-acetylcholine release (Milenov *et al.*, 1991) while in LMMP preparations of guinea-pig ileum, VIP and PACAP increased basal and inhibited electrically-induced [^{3}H]-acetylcholine release from cholinergic nerve endings (Katsoulis *et al.*, 1993). Also in ferret trachea, VIP inhibited [^{3}H]-acetylcholine release from cholinergic neurones regulating mucus secretion (Liu *et al.*, 1999). In human proximal stomach, we did not obtain evidence that VIP inhibits electrically-induced [^{3}H]-acetylcholine release, suggesting that VIP and acetylcholine only functionally antagonise each other when released simultaneously. This is in agreement

with observations in rat gastric fundus and trachea, and human airway (Lefebvre *et al.*, 1992; Sekizawa *et al.*, 1993; Ward *et al.*, 1993).

Noradrenaline is able to inhibit non-sphincteric muscle in the GI tract by inhibition of acetylcholine release from the cholinergic motor neurones *via* presynaptic α_2-adrenoceptors (see reviews McIntyre & Thompson, 1992; De Ponti *et al.*, 1996). In pig gastric fundus, noradrenaline and adrenaline have been shown to produce concentration-dependent contractions due to activation of postsynaptic α-adrenoceptors (Mandrek & Kreis, 1992). A possible inhibitory effect of α-adrenoceptor agonists on electrically-induced cholinergic contractions in contraction experiments might thus be masked by the contractile effect *per se* of these substances. In preliminary experiments (data not shown), we observed that addition of the α_2-adrenoceptor agonist UK-14,304 induced contractions of circular smooth muscle strips in the pig gastric fundus, suggesting that α_2-adrenoceptors are present on the circular smooth muscle cells of pig gastric fundus. We thus investigated the effect of UK-14,304 on the electrically-induced release of [^{3}H]-acetylcholine. UK-14,304 inhibited the electrically-evoked [^{3}H]-acetylcholine release in pig gastric fundus, an effect that was prevented by the α_2-adrenoceptor antagonist rauwolscine. Also in human gastric fundus, the electrically-induced [^{3}H]-acetylcholine release was inhibited by stimulation of α_2-adrenoceptors, the degree of inhibition being even larger than in pig gastric fundus. Thus, presynaptic inhibitory α_2-adrenoceptors are present on the cholinergic nerve endings innervating the circular muscle layer of pig and human gastric fundus, corresponding with results obtained in other parts of the GI tract (McIntyre & Thompson, 1992; De Ponti *et al.*, 1996).

Stimulation of 5-HT$_4$-receptors induces contraction or relaxation depending on the tissue and species studied. Relaxant 5-HT$_4$-receptors are localized on smooth muscle cells (see e.g. Tuladhar *et al.*, 1996; Prins *et al.*, 2000b), while contractile responses are ascribed to 5-HT$_4$-receptors localized on cholinergic neurones, facilitating acetylcholine release (see e.g. Elswood *et al.*, 1991; Kilbinger & Wolf, 1992). In rat gastric fundus, facilitatory 5-HT$_4$-receptors have been suggested to be present on cholinergic neurones, increasing electrically-evoked cholinergic contractions (Amemiya *et al.*, 1996). Matsuyama and colleagues (1996) demonstrated that 5-HT$_4$-receptor agonists enhanced electrically-induced cholinergic contractions and [^{3}H]-acetylcholine release in strips from all regions of guinea-pig stomach. In a more recent study by Takada and colleagues (1999), a regional distribution of facilitatory 5-HT$_4$-receptors on cholinergic neurones innervating the guinea-pig stomach was suggested whereby neuronal 5-HT$_4$-receptors seem to be present in the corpus and antrum but absent in

the fundus. In human, the 5-HT_4-receptor agonist cisapride increased solid and liquid gastric emptying *in vivo* (Johnson, 1989). Prucalopride, another 5-HT_4-receptor agonist, had no effect on gastric emptying in healthy humans (Bouras *et al.*, 1999), but it accelerated gastric emptying in patients with constipation (Bouras *et al.*, 2001). *In vitro*, prucalopride enhanced electrically-induced contractions of circular muscle strips of human stomach (Ver Donck *et al.*, 1999), but the precise mechanism of this stimulatory effect was not investigated. Our aim was to study the mechanism of the stimulatory effect of prucalopride in human gastric fundus. We observed in our study a stimulatory effect of prucalopride on electrically-induced [^{3}H]-acetylcholine release in human gastric fundus. This effect was prevented by the 5-HT_4-receptor antagonist SB204070, strengthening the idea that facilitatory 5-HT_4-receptors are also present on the cholinergic nerve endings innervating the circular muscle layer of human gastric fundus. Stimulation of these receptors by 5-HT_4-receptor agonists will contribute to the stimulatory effect of these agents on gastric emptying.

An integrative scheme of our results on modulation of cholinergic neurotransmission in the gastric fundus, discussed in 7.1, 7.2 and 7.3, is shown in Figure 7.1. Selective α_2-adrenoceptor agonists and 5-HT_4-receptor agonists might be used to inhibit respectively stimulate cholinergic neurotransmission in the proximal stomach as required. The similarity of the results in the pig and human gastric fundus illustrates that the pig is a good model to study the regulation of human gastric motility.

7.4. Presynaptic modulation of acetylcholine release *via* 5-HT_4-receptors in the human colon

In vivo studies in humans demonstrated that prucalopride stimulates colonic transit (Emmanuel *et al.*, 1998; Bouras, 1999, 2001). In dog, prucalopride stimulated proximal, and inhibited distal colonic motility, and reduced the time to the first GMC (Briejer *et al.*, 2001). *In vitro* experiments demonstrated that in the longitudinal muscle layer of both canine large intestine (from ascending to descending colon) and human large intestine (from ascending colon to rectum), 5-HT_4-receptors on cholinergic neurones mediate facilitation of cholinergic neurotransmission resulting in enhanced longitudinal muscle contractility (Prins *et al.*, 2000a) (Figure 7.2). This correlates with facilitatory 5-HT_4-receptors on cholinergic neurones in LMMP preparations of guinea-pig proximal colon (Elswood *et al.*, 1991; Briejer &

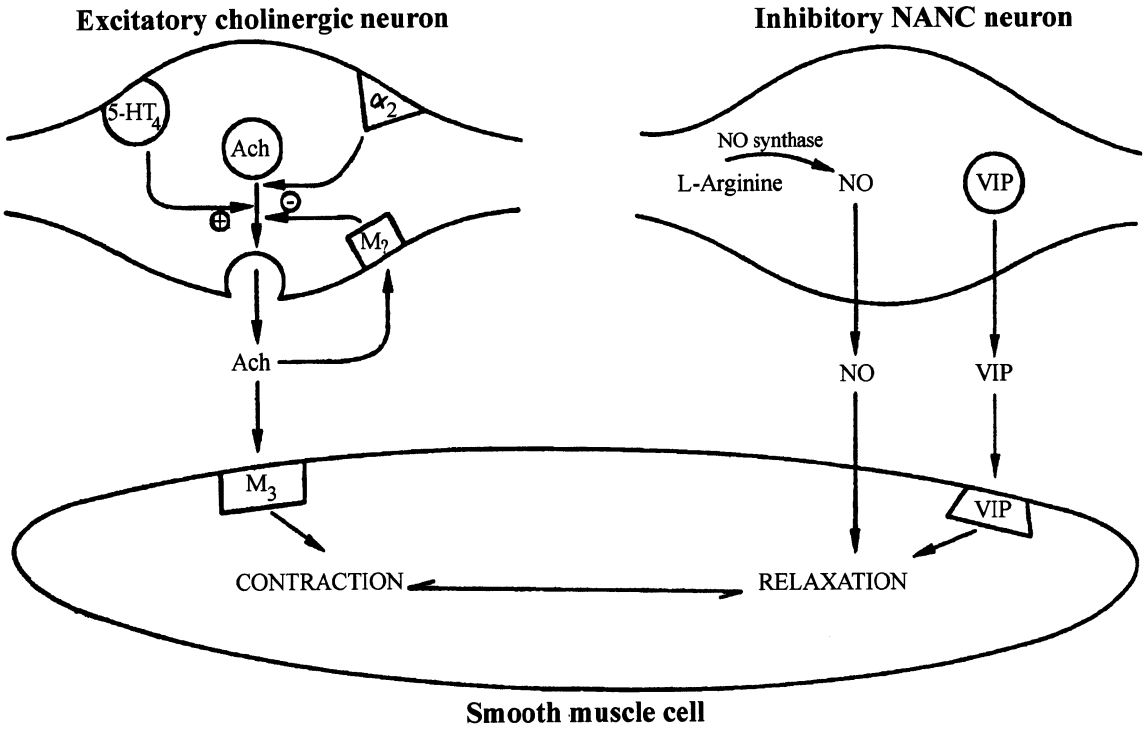

Figure 7.1 Integrative scheme summarising our results on modulation of cholinergic neurotransmission in the gastric fundus. Acetylcholine (ACh) is released and interacts with postsynaptic M_3 receptors to induce contraction. It can exert negative feedback on its release *via* presynaptic muscarinic receptors; the latter cannot be pharmacologically characterised within the M_1-M_5 classification. Nitric oxide (NO) and vasoactive intestinal polypeptide (VIP), released from non-adrenergic non-cholinergic (NANC) neurones functionally antagonise acetylcholine at the smooth muscle cells. The release of acetylcholine can be stimulated *via* presynaptic 5-HT_4-receptors (5-HT_4) and inhibited *via* presynaptic α_2-adrenoceptors (α_2).

Schuurkes, 1996). In canine large intestine circular muscle, smooth muscle 5-HT_4-receptors mediate relaxation, a phenomenon that gradually increases in efficiency going from the ascending colon to the rectum (Prins *et al.*, 1999). In human large intestine circular muscle, 5-HT_4-receptors located on smooth muscle mediate relaxation, with a similar efficiency throughout the large intestine (Tam *et al.*, 1994; Prins *et al.*, 2000b) (Figure 7.2). This inhibitory action on circular muscle tone demonstrated *in vitro* may explain the distally increasing circular muscle inhibition observed with prucalopride in dog large intestine *in vivo* (Briejer *et al.*, 2001). The stimulatory effect of prucalopride on human colonic transit can very probably not only be explained by interaction with the relaxant 5-HT_4-receptors on the circular smooth muscle cells and the 5-HT_4-receptors on the cholinergic nerve endings innervating the longitudinal muscle layer. Indeed, although the circular muscle relaxation might induce a decrease in luminal resistance towards transit, it is unlikely that the three bands of longitudinal muscle can provide sufficient contractile force to promote transit.

However, a coordinated mechanism whereby the circular muscle first relaxes and then is activated *via* its cholinergic innervation might explain the effect of prucalopride on colonic transit. For this reason, the presence of 5-HT_4-receptors on the cholinergic neurones innervating the circular muscle layer was investigated.

Therefore, the effect of prucalopride on electrically-induced [^{3}H]-acetylcholine release was studied. In whole tissue strips, cut in the direction of the circular muscle, prucalopride increased electrically-induced [^{3}H]-acetylcholine release and this effect was antagonised by the 5-HT_4-receptor antagonist GR113808. However, whole tissue circular muscle strips contain some longitudinal muscle material. This means that the cholinergic nerve endings innervating the longitudinal muscle layer can contribute to some extent to the amount of [^{3}H]-acetylcholine detected. As facilitatory 5-HT_4-receptors have been described on the cholinergic nerve endings innervating the longitudinal muscle layer in the human colon (Prins *et al.*, 2000a), the effect of prucalopride on the electrically-induced [^{3}H]-acetylcholine release

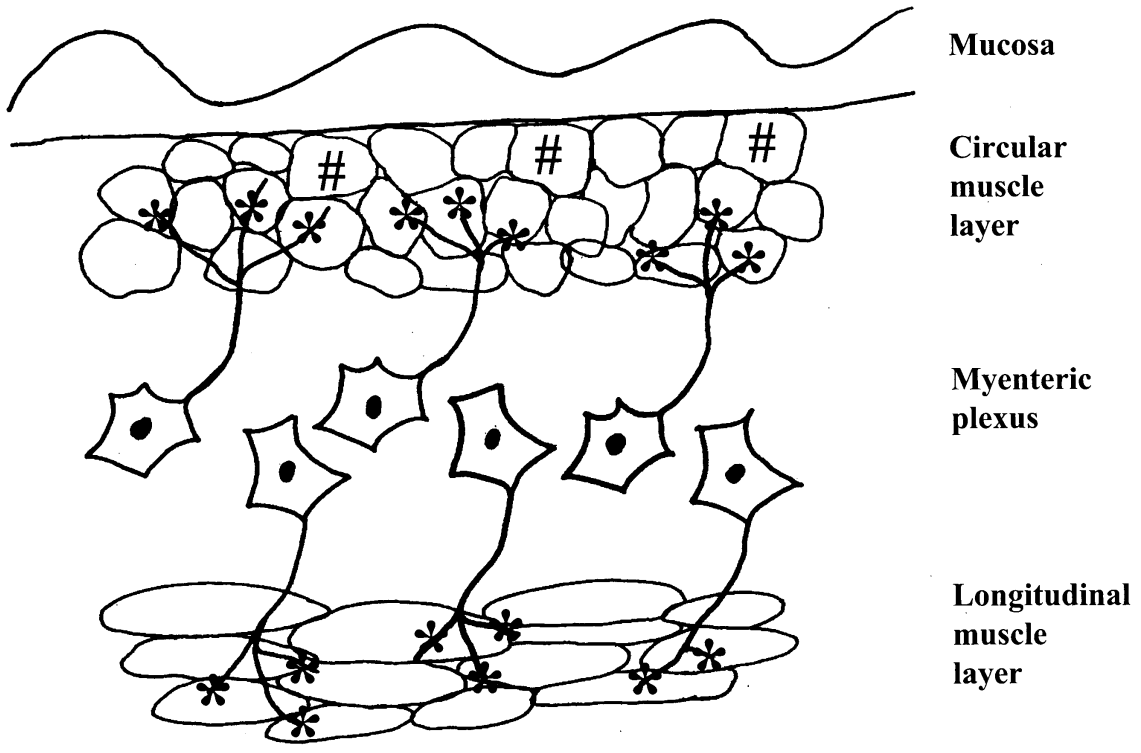

Figure 7.2 Schematic representation of the locations of 5-HT_4-receptors in human colon. Muscular 5-HT_4-receptors (#) are present on the smooth muscle cells of the circular muscle layer, causing relaxation (Prins *et al.*, 2000b). Neuronal 5-HT_4-receptors (*) are shown to facilitate acetylcholine release from cholinergic nerve endings innervating the longitudinal (Prins *et al.*, 2000a) and circular (this thesis) muscle layer.

in the whole tissue strips might be due to activation of these 5-HT_4-receptors. For this reason, strips only containing part of the circular muscle layer and its nerve endings were prepared. Prucalopride enhanced the electrically-induced [^{3}H]-acetylcholine release in these isolated circular muscle strips. This effect was antagonised by the 5-HT_4-receptor antagonist GR113808. Our results thus clearly demonstrate that 5-HT_4-receptors are present on the cholinergic nerve endings innervating the circular muscle layer in the human colon (Figure 7.2). Contraction experiments with isolated circular muscle strips were not feasible because of very weak contractile responses to electrical stimulation. When the influence of prucalopride was studied on electrically-induced cholinergic contractions in whole tissue strips cut in the circular direction, its effect on these electrically-induced cholinergic contractions varied from potentiation to inhibition. Any kind of effect of prucalopride was antagonised by GR113808, indicating that the action of prucalopride was due to activation of 5-HT_4-receptors. In one tissue, prucalopride enhanced the electrically-induced contractions, supporting the results from our release study that facilitatory 5-HT_4-receptors are present on the cholinergic neurones innervating the circular muscle layer. However, in four tissues, prucalopride inhibited the electrically-evoked contractions, probably *via* activation of the relaxant 5-HT_4-receptors on the circular muscle cells and functional antagonism of acetylcholine released. In three other tissues, prucalopride had no effect on electrically-induced contractions. The influence of prucalopride on electrically-induced cholinergic contractions in whole tissue strips is the balance of the activation of the inhibitory 5-HT_4-receptors on the circular smooth muscle cells and that of the stimulatory 5-HT_4-receptors on the cholinergic nerve endings towards the circular muscle. The variability of effect of prucalopride might be explained by the varying density of neuronal *versus* muscular 5-HT_4-receptors across specimens.

Figure 7.2 gives a schematic representation of the locations of 5-HT_4-receptors in human colon. Muscular 5-HT_4-receptors can be found on circular smooth muscle cells (Prins *et al.*, 2000b). Facilitatory 5-HT_4-receptors are present on cholinergic nerve endings innervating the longitudinal (Prins *et al.*, 2000a) and circular (this thesis) muscle layer. The effect of prucalopride on human colonic transit might be due to the coordinated stimulation of inhibitory 5-HT_4-receptors on the circular muscle cells and facilitatory neuronal 5-HT_4-receptors on cholinergic neurones innervating the circular muscle, whereby the circular muscle of the colon first relaxes and then is activated *via* its cholinergic innervation. The relaxation of the colon allows an easy propagation of the faecal contents by the contraction.

7.4 References

ADEM, A. & KARLSSON, E. (1997). Muscarinic receptor subtype selective toxins. *Life Sciences*, **60**, 1069-1076

AMEMIYA, N., HATTA, S., TAKEMURA, H. & OHSHIKA, H. (1996). Characterization of the contractile response induced by 5-methoxytryptamine in rat stomach fundus strips. *Eur. J. Pharmacol.*, **318**, 403-409

BACCARI, M.C., CALAMAI, F. & STADERINI, G. (1994). Modulation of cholinergic transmission by nitric oxide, VIP and ATP in the gastric muscle. *Neuroreport*, **5**, 905-908

BELLIDO, I., FERNANDEZ, J.L., GOMEZ, A. & SANCHEZ DE LA CUESTA, F. (1995). Otenzepad shows two populations of binding sites in human gastric smooth muscle. *Can J. Physiol. Pharmacol.*, **73**, 124-129

BOURAS, E.P., CAMILLERI, M., BURTON, D.D. & MCKINZIE, S. (1999). Selective stimulation of colonic transit by the benzofuran $5HT_4$ agonist, prucalopride, in healthy humans. *Gut*, **44**, 682-686

BOURAS, E.P., CAMILLERI, M., BURTON, D.D., THOMFORDE, G., MCKINZIE, S. & ZINSMEISTER, A.R. (2001). Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. *Gastroenterology*, **120**, 354-360

BRAVE, S.R., HOBBS, A.J., GIBSON, A. & TUCKER, J.F. (1991). The influence of L-N^G-nitro-arginine on field stimulation induced contractions and acetylcholine release in guinea pig isolated tracheal smooth muscle. *Biochem. Biophys. Res. Comm.*, **179**, 1017-1022

BRIEJER, M.R., PRINS, N.H. & SCHUURKES, J.A.J. (2001). Effects of the enterokinetic prucalopride (R093877) on colonic motility in fasted dogs. *Neurogastroenterol. Mot.*, **13**, 465-472

BRIEJER, M.R. & SCHUURKES, J.A.J. (1996). 5-HT_3 and 5-HT_4 receptors and cholinergic and tachykininergic neurotransmission in the guinea-pig proximal colon. *Eur. J. Pharmacol.*, **308**, 173-180

CAULFIELD, M.P. & BIRDSALL, N.J.M. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.*, **50**, 279-290

CELLEK, S. & MONCADA, S. (1997). Nitrergic modulation of cholinergic responses in the opossum lower oesophageal sphincter. *Br. J. Pharmacol.*, **122**, 1043-4046

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.*, **69**, 59-78

DIETRICH, C. & KILBINGER, H. (1995). Prejunctional M1 and postjunctional M3 muscarinic receptors in the circular muscle of the guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **351**, 237-243

EGLEN, R.M., HEDGE, S.S. & WATSON, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.*, **48**, 531-565

ELSWOOD, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Identification of putative 5-HT_4-receptors in guinea-pig ascending colon. *Eur. J. Pharmacol.*, **196**, 149-155

EMMANUEL, A.V., KAMM, M.A., ROY, A.J. & ANTONELLI, K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut*, **42**, 511-516

FURNESS, J.B., BORNSTEIN, J.C., MURPHY, R. & POMPOLO, S. (1992). Roles of peptides in transmission in the enteric nervous system. *Trends Neurosci.*, **15**, 361-372

GRIMM, U., MOSER, E., MUTSCHLER, M.E. & LAMBRECHT, G. (1994). Muscarinic receptors: focus on presynaptic mechanisms and recently developed novel agonists and antagonists. *Pharmazie*, **49**, 711-726

HEBEIβ, K. & KILBINGER, H. (1996). Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. *Br. J. Pharmacol.*, **118**, 2073-2078

HEBEISS, K. & KILBINGER, H. (1998). Nitric oxide-sensitive guanylyl cyclase inhibits acetylcholine release and excitatory motor transmission in the guinea-pig ileum. *Neuroscience*, **82**, 623-629

HERAWI, M., LAMBRECHT, G., MUTSCHLER, E., MOSER, U. & PFEIFFER, A. (1988). Different binding properties of muscarinic M_2-receptor subtypes for agonists and antagonists in porcine gastric smooth muscle and mucosa. *Gastroenterology*, **94**, 630-637

HRYHORENKO, L.M., WOSKOWSKA, Z. & FOX-THRELKELD, J.-A.E.T. (1994). Nitric oxide (NO) inhibits release of acetylcholine from nerves of isolated circular muscle of the canine ileum: relationship to motility and release of nitric oxide. *J. Pharmacol. Exp. Ther.*, **271**, 918-926

JERUSALINSKY, D., KORNISIUK, E., ALFARO, P., QUILLFELDT, J., FERREIRA, A., RIAL, V.E., DURAN, R. & CERVENANSKY, C. (2000). Muscarinic toxins: novel pharmacological tools for the muscarinic cholinergic system. *Toxicon*, **38**, 747-761

JOHNSON, A.G. (1989). The effects of cisapride on antroduodenal co-ordination and gastric emptying. *Scand. J. Gastroenterol.*, **24**, 36-43

KATSOULIS, S., CLEMENS, A., SCHWÖRER, H., CREUTZFELDT, W. AND SCHMIDT, W.E. (1993). PACAP is a stimulator of neurogenic contraction in guinea pig ileum. *Am. J. Physiol.*, **265**, G295-G302

KILBINGER, H. & WOLF, D. (1992). Effects of 5-HT_4 receptor stimulation on basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **345**, 270-275

KILBINGER, H. & WOLF, D. (1994). Increase by NO synthase inhibitors of acetylcholine release from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349**, 543-545

KOSTKA, P., KWAN, C.-Y. & DANIEL, E.E. (1989). Presynaptic and postsynaptic muscarinic receptors in dog ileum: binding studies. *Eur. J. Pharmacol.*, **173**, 35-42

LEFEBVRE, R.A., DE VRIESE, A. & SMITS, G.J.M. (1992). Influence of vasoactive intestinal polypeptide and N^G-nitro-L-arginine methyl ester on cholinergic neurotransmission in the rat gastric fundus. *Eur. J. Pharmacol.*, **221**, 235-242

LEFEBVRE, R.A., SMITS, G.J.M. & TIMMERMANS, J.-P. (1995). Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. *Br. J. Pharmacol.*, **116**, 2017-2026

LIU, Y.C., PATEL, H.J., KHAWAJA, A.M., BELVISI, M.G. & ROGERS, D.F. (1999). Neuroregulation by vasoactive intestinal peptide (VIP) of mucus secretion in ferret trachea: activation of BK_{Ca} channels and inhibition of neurotransmitter release. *Br. J. Pharmacol.*, **126**, 147-158

MANDREK, K. & KREIS, S. (1992). Regional differentiation of gastric and of pyloric smooth muscle in the pig: mechanical responses to acetylcholine, histamine, substance P, noradrenaline and adrenaline. *J. Auton. Pharmacol.*, **12**, 37-49

MANG, C.F., TRÜMPLER, S. & KILBINGER, H. (2000). Inhibition by endogenous nitric oxide of acetylcholine release in the mouse isolated ileum. *Br. J. Pharmacol.*, **131**, 32P

MATSUYAMA, S., SAKIYAMA, H., NEI, K. & TANAKA, C. (1996). Identification of putative 5-hydroxytryptamine$_4$ (5-HT_4) receptors in guinea pig stomach: the effect of TKS159, a novel agonist, on gastric motility and acetylcholine release. *J. Pharmacol. Exp. Ther.*, **276**, 989-995

MCINTYRE, A.S. & THOMPSON, D.G. (1992). Review article: adrenergic control of motor and secretory function in the gastrointestinal tract. *Aliment. Pharmacol. Ther.*, **6**, 125-142

MILENOV, K. & KALFIN, R. (1996). Cholinergic -nitrergic interactions in the guinea-pig gastric fundus. *Neuropeptides*, **30**, 365-371

MILENOV, K., KALFIN, R. & MANDREK, K. (1991). Effect of vasoactive intestinal peptide (VIP) on the mechanical activity and [^{3}H] acetylcholine release in guinea-pig gastric muscle. *Acta Physiol. Pharmacol. Bulg.*, **17**, 13-18

OLIANAS, M.C., INGIANNI, A., MAULLU, C., ADEM, A., KARLSSON, E. & ONALI, P. (1999). Selectivity profile of muscarinic toxin 3 in functional assays of cloned and native receptors. *J. Pharmacol. Exp. Ther.*, **288**, 167-170

PREIKSAITIS, H.G. & LAURIER, L.G. (1998). Pharmacological and molecular characterization of muscarinic receptors in cat esophageal smooth muscle. *J. Pharmacol. Exp. Ther.*, **285**, 853-861

PRINS, N.H., VAN HASELEN, J.F.W.R., LEFEBVRE, R.A., BRIEJER, M.R., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (1999). Pharmacological characterisation of canine isolated rectum circular smooth muscle. *Br. J. Pharmacol.*, **127**, 1431-1437

PRINS, N.H., AKKERMANS, L.M.A., LEFEBVRE, R.A. & SCHUURKES, J.A.J. (2000a). 5-HT$_4$ receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br. J. Pharmacol.*, **131**, 927-932

PRINS, N.H., SHANKLEY, N.P., WELSH, N.J., BRIEJER, M.R., LEFEBVRE, R.A., AKKERMANS, L.M.A. & SCHUURKES, J.A.J. (2000b). An improved *in vitro* bioassay for the study of 5-HT$_4$ receptors in the human isolated large intestinal circular muscle. *Br. J. Pharmacol.*, **129**, 1601-1608

SEKIZAWA, K., FUKUSHIMA, T., IKARASHI, Y., MARUYAMA, Y. & SASAKI, H. (1993). The role of nitric oxide in cholinergic neurotransmission in rat trachea. *Br. J. Pharmacol.*, **110**, 816-820

SHI, X.-Z. & SARNA, S.K. (1997). Inflammatory modulation of muscarinic receptor activation in canine ileal circular muscle cells. *Gastroenterology*, **112**, 864-874

SOEJIMA, O., KATSURAGI, T. & FURUKAWA, T. (1993). Opposite modulation by muscarinic M_1 and M_3 receptors of acetylcholine release from guinea pig ileum as measured directly. *Eur. J. Pharmacol.*, **249**, 1-6

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 864-989

TAKADA, K., SAKURAI-YAMASHITA, Y., YAMASHITA, K., KAIBARA, M., HAMADA, Y., NAKANE, Y., HIOKI, K. & TANIYAMA, K. (1999). Regional difference in correlation of 5-HT_4-receptor distribution with cholinergic transmission in the guinea-pig stomach. *Eur. J. Pharmacol.*, **374**, 489-494

TAM, F.S.-F., HILLIER, K. & BUNCE, K.T. (1994). Characterization of the 5-hydroxytryptamine receptor type involved in inhibition of spontaneous activity of human isolated colonic circular muscle. *Br. J. Pharmacol.*, **113**, 143-150

TONINI, M., DE GIORGIO, R., DE PONTI, F., STERNINI, C., SPELTA, V., DIONIGI, P., BARBARA, G., STANGHELLINI, V. & CORINALDESI, R. (2000). Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations. *Br. J. Pharmacol.*, **129**, 12-20

TULADHAR, B.R., COSTALL, B. & NAYLOR, R.J. (1996). Pharmacological characterization of the 5-hydroxytryptamine receptor mediating relaxation in the rat isolated ileum. *Br. J. Pharmacol.*, **119**, 303-310

VER DONCK, L., MEULEMANS, A.L., PRINS, N.H. & SCHUURKES, J.A.J. (1999). Prucalopride enhances contractions of the electrically stimulated human stomach *via* 5-HT_4 receptor activation. *Neurogastroenterol. Mot.*, **11**, 299

WARD, J.K., BELVISI, M.G., FOX, A.J., MIURA, M., TADJKARIMI, S., YACOUB, M. & BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. *J. Clin. Invest.*, **92**, 736-743

WIKLUND, C.U., OLGART, C., WIKLUND, N.P. & GUSTAFSSON, L.E. (1993). Modulation of cholinergic and substance P-like neurotransmission by nitric oxide in the guinea-pig ileum. *Br. J. Pharmacol.*, **110**, 833-839

SUMMARY

SUMMARY

Gastrointestinal (GI) motility is under neuronal and hormonal control. The acetylcholine-releasing cholinergic neurones are the most important among the neurones that release a contractile neurotransmitter; in contrast, nitric oxide (NO) is the most important relaxant neurotransmitter. Studies in the peripheral and central nervous system demonstrated that the release of acetylcholine from cholinergic nerve endings can be modified *via* presynaptic auto- and heteroreceptors, whereby the release of acetylcholine can be increased or inhibited. In the GI tract, there is some evidence that NO modifies the release of acetylcholine presynaptically, while other authors argue against a modulatory role for NO at the presynaptic level and suggest that NO only functionally antagonises acetylcholine. The sympathetic nervous system, releasing noradrenaline, is known to modulate acetylcholine release in non-sphincteric regions of the GI tract. 5-HT_4-receptors, stimulating acetylcholine release, have been demonstrated on cholinergic nerve endings in the GI tract, although also relaxant 5-HT_4-receptors located on smooth muscle cells have been described. To investigate the presynaptic regulation of acetylcholine release, a technique measuring acetylcholine release directly must be applied.

The aim of this thesis was to study the presynaptic regulation of acetylcholine release in the porcine and human gastric fundus. In human colon, the presence of 5-HT_4-receptors on the cholinergic nerve endings innervating the circular muscle layer was investigated.

The first study investigated the functional interaction between the cholinergic and nitrergic innervation in circular muscle strips of the pig gastric fundus (**Chapter 2**). Both cholinergic and nitrergic neurones are stimulated during electrical stimulation; the nitrergic contribution is dominant at lower and the cholinergic contribution at higher stimulation frequencies. The results suggest that the interaction between NO and acetylcholine lies at the postsynaptic level but a presynaptic effect of NO on acetylcholine release could not be fully excluded.

A method was therefore introduced to measure acetylcholine release from the cholinergic nerve endings after incubation of the tissue with [^{3}H]-choline (**Chapter 3**). Electrical stimulation of circular muscle strips of the pig gastric fundus activated cholinergic neurones causing the release of [^{3}H]-acetylcholine, although stimulation also induced some [^{3}H]-phosphorylcholine and [^{3}H]-choline release. Neither the NO synthase inhibitor L-N^G-nitro-arginine methyl ester (L-NAME) nor the NO donors sodium nitroprusside (SNP) and 3-

morpholino-sydnonimine (SIN-1) had any effect on [^{3}H]-acetylcholine release, suggesting that NO does not interfere with the release of [^{3}H]-acetylcholine from cholinergic neurones innervating the circular muscle layer of pig gastric fundus. The α_2-adrenoceptor agonist UK-14,304 inhibited the electrically-induced [^{3}H]-acetylcholine release, an effect prevented by the α_2-adrenoceptor antagonist rauwolscine, suggesting that inhibitory α_2-adrenoceptors are present on the cholinergic neurones. The non-selective muscarinic antagonist atropine increased stimulation-induced [^{3}H]-acetylcholine release, suggesting the presence of inhibitory muscarinic autoreceptors on the cholinergic neurones.

An attempt was made to characterize pharmacologically the presynaptic muscarinic receptors on the cholinergic nerve endings in pig gastric fundus by using a series of subtype-preferring muscarinic antagonists (**Chapter 4**). However, we were not able to classify the presynaptic muscarinic receptor, although the presynaptic presence of the M_2 subtype could be excluded. A second goal was the pharmacological characterization of the postsynaptic muscarinic receptors. Acetylcholine, released upon stimulation, is responsible for the contraction of the tissue after activation of muscular M_3 receptors; no evidence for the occurrence of another muscular muscarinic receptor was found.

Also in human gastric fundus, [^{3}H]-acetylcholine release from cholinergic neurones innervating the circular muscle layer was measured (**Chapter 5**). The increase in radioactivity during stimulation was again mainly due to [^{3}H]-acetylcholine but to some extent also to [^{3}H]-phosphorylcholine and [^{3}H]-choline. In human gastric fundus, neither NO nor VIP inhibited the electrically-induced [^{3}H]-acetylcholine release, indicating that these two neurotransmitters only functionally antagonise acetylcholine. UK-14,304 inhibited electrically-induced [^{3}H]-acetylcholine release, an effect prevented by the α_2-adrenoceptor antagonist rauwolscine, suggesting the presence of presynaptic inhibitory α_2-adrenoceptors on the cholinergic nerve endings. Atropine increased the electrically-induced [^{3}H]-acetylcholine release, indicative for the presence of presynaptic inhibitory autoreceptors. The 5-HT$_4$-receptor agonist prucalopride stimulated the electrically-induced [^{3}H]-acetylcholine release, an effect that was prevented by the 5-HT$_4$-receptor antagonist SB204070, suggesting the presence of facilitatory 5-HT$_4$-receptors on the cholinergic nerve endings innervating the circular muscle layer.

As the stimulatory effect of 5-HT$_4$-receptor agonists on colonic transit *in vivo* cannot be fully explained by interaction with the 5-HT$_4$-receptors that were described in the human colon *in vitro*, we studied whether 5-HT$_4$-receptors are present on the cholinergic nerve

endings innervating the circular muscle layer in the human colon (**Chapter 6**). When the effect of prucalopride was measured on electrically-induced [^{3}H]-acetylcholine release in tissues cut in the direction of the circular muscle layer (these contain besides of circular muscle also some longitudinal muscle material and the myenteric plexus; whole tissue), as well as in strips containing only isolated circular muscle with its nerve endings, we observed that prucalopride enhanced electrically-induced [^{3}H]-acetylcholine release; the 5-HT_4-receptor antagonist GR113808 antagonised this effect, supportive of the presence of stimulatory 5-HT_4-receptors on cholinergic nerve endings innervating the circular muscle layer in human colon. When the influence of prucalopride was studied on electrically-induced cholinergic contractions in whole tissue strips, they were either potentiated, not influenced or even inhibited. This can probably be ascribed by a variable balance between the stimulatory 5-HT_4-receptors on the cholinergic neurones, described in this thesis, and the inhibitory 5-HT_4-receptors on the smooth muscle cells in the human colon, described before.

In conclusion, our results show that the pig gastric fundus is a good model to study the human gastric fundus. Both NO and VIP only postsynaptically antagonise acetylcholine at this level of the GI tract. The cholinergic neurones from porcine and human gastric fundus contain presynaptic inhibitory muscarinic autoreceptors and α_2-adrenoceptors. Presynaptic stimulatory 5-HT_4-receptors are present on cholinergic nerve endings innervating the circular muscle layer of human gastric fundus as well as of human colon.

SAMENVATTING

SAMENVATTING

Gastro-intestinale (GI) motiliteit staat onder neuronale en hormonale controle. De cholinerge zenuwcellen, die acetylcholine vrijstellen, zijn de belangrijkste zenuwcellen die een contractiele neurotransmitter vrijstellen; daartegenover staat stikstofmonoxide (NO) als belangrijkste relaxerende neurotransmitter. Studies, uitgevoerd in het perifere en centrale zenuwstelsel, toonden aan dat de vrijstelling van acetylcholine uit cholinerge zenuwuiteinden beïnvloed kan worden *via* presynaptische auto- en heteroreceptoren, waarbij de vrijstelling van acetylcholine gestimuleerd of verminderd kan worden. Sommige resultaten suggereren dat NO de vrijstelling van acetylcholine presynaptisch kan beïnvloeden in het GI kanaal; andere auteurs argumenteren dat NO geen regulerende rol vervult op het presynaptische niveau en suggereren dat NO acetylcholine enkel functioneel tegenwerkt. Van het sympathische zenuwstelsel, dat noradrenaline vrijstelt, is geweten dat het de vrijstelling van acetylcholine kan beïnvloeden in de sphinctervrije zones van het GI kanaal. 5-HT_4-receptoren die de vrijstelling van acetylcholine stimuleren, zijn aangetoond op de cholinerge zenuwuiteinden in het GI kanaal, hoewel er ook relaxerende 5-HT_4-receptoren op gladde spiercellen beschreven zijn. Ten einde de presynaptische regeling van de vrijstelling van acetylcholine te onderzoeken, moet een techniek worden toegepast die de vrijstelling van acetylcholine rechtstreeks meet.

Het doel van deze thesis bestond uit het bestuderen van de presynaptische regeling van de vrijstelling van acetylcholine in de maagfundus van het varken en de mens. In menselijk colon werd de aanwezigheid van 5-HT_4-receptoren op cholinerge zenuwuiteinden naar de circulaire spierlaag onderzocht.

De eerste studie onderzocht de functionele interactie tussen de cholinerge en nitrerge bezenuwing van circulaire spierstrips van de maagfundus van het varken (**Hoofdstuk 2**). Zowel cholinerge als nitrerge zenuwcellen worden geactiveerd tijdens elektrische stimulatie; de nitrerge bijdrage is de belangrijkste tijdens lagere and de cholinerge tijdens hogere stimulatie-frequenties. De resultaten suggereren dat de interactie tussen NO en acetylcholine op het postsynaptische niveau gelegen is maar een presynaptisch effect van NO op de vrijstelling van acetylcholine kon niet worden uitgesloten.

Daarom werd een methode geïntroduceerd om de vrijstelling van acetylcholine van cholinerge zenuwuiteinden te meten na incubatie van de weefsels met [^{3}H]-choline (**Hoofdstuk 3**). Elektrische stimulatie van de circulaire spierstrips van de maagfundus van

het varken stimuleerde cholinerge zenuwcellen met als gevolg de vrijstelling van [^{3}H]-acetylcholine, hoewel stimulatie ook enige vrijstelling van [^{3}H]-phosphorylcholine en [^{3}H]-choline uitlokte. Noch de NO-synthase-inhibitor L-N^G-nitro-arginine methyl ester (L-NAME) noch de NO-donoren natriumnitroprusside (SNP) en 3-morfolino-sydnonimine (SIN-1) beïnvloedden de vrijstelling van [^{3}H]-acetylcholine, wat suggereert dat NO niet interfereert met de vrijstelling van acetylcholine uit cholinerge zenuwuiteinden die de circulaire spierlaag van de maagfundus van het varken bezenuwen. De α_2-adrenoceptor agonist UK-14,304 verminderde de elektrisch geïnduceerde [^{3}H]-acetylcholine vrijstelling, een effect dat voorkomen werd door de α_2-adrenoceptor antagonist rauwolscine, wat suggereert dat remmende α_2-adrenoceptoren aanwezig zijn op de cholinerge zenuwuiteinden. De niet-selectieve muscarine-antagonist atropine veroorzaakte een toename van de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine wat suggereert dat remmende muscarine-autoreceptoren aanwezig zijn op de cholinerge zenuwcellen.

Een poging werd ondernomen om farmacologisch de presynaptische muscarine-receptoren op de cholinerge zenuwuiteinden in de maagfundus van het varken te karakteriseren door gebruik te maken van een reeks muscarine-antagonisten met een specificiteit voor een bepaald subtype (**Hoofdstuk 4**). We waren niet in staat om de presynaptische muscarinereceptor te bepalen, hoewel de presynaptische aanwezigheid van het M_2 subtype uitgesloten kon worden. Een tweede doelstelling was het farmacologisch typeren van de postsynaptische muscarinereceptoren. Acetylcholine, vrijgesteld tijdens stimulatie, is verantwoordelijk voor de contractie van het weefsel na activatie van M_3 receptoren gelegen op de spiercellen; er werd geen evidentie voor de aanwezigheid van een andere muscarine-receptor op de spiercellen gevonden.

Ook in de maagfundus van de mens werd de vrijstelling van [^{3}H]-acetylcholine uit cholinerge zenuwcellen die de circulaire spierlaag bezenuwen gemeten (**Hoofdstuk 5**). De toename van de radioactiviteit tijdens stimulatie was opnieuw vooral het gevolg van [^{3}H]-acetylcholine maar in bepaalde mate ook van [^{3}H]-phosphorylcholine en [^{3}H]-choline. In de maagfundus van de mens verminderde noch NO noch VIP de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine wat aantoont dat deze twee neurotransmitters acetylcholine enkel functioneel tegenwerken. UK-14,304 verminderde de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine, een effect dat voorkomen werd door de α_2-adrenoceptor antagonist rauwolscine, wat suggereert dat presynaptische remmende α_2-adrenoceptoren aanwezig zijn op de cholinerge zenuwuiteinden. Atropine deed de elektrisch geïnduceerde

vrijstelling van [^{3}H]-acetylcholine toenemen, wat de aanwezigheid van presynaptische remmende autoreceptoren aantoont. De 5-HT$_4$-receptor agonist prucalopride stimuleerde de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine, een effect dat door de 5-HT$_4$-receptor antagonist SB204070 voorkomen werd, wat suggereert dat stimulerende 5-HT$_4$-receptoren aanwezig zijn op de cholinerge zenuwuiteinden die de circulaire spierlaag bezenuwen.

Vermits het stimulerend *in vivo* effect van 5-HT$_4$-receptor agonisten op de doorgang in het colon niet volledig verklaard kan worden door een interactie met de 5-HT$_4$-receptoren beschreven in het menselijk colon *in vitro*, hebben we de aanwezigheid van 5-HT$_4$-receptoren op de cholinerge zenuwuiteinden naar de circulaire spierlaag van het menselijk colon functioneel onderzocht (**Hoofdstuk 6**). Wanneer het effect van prucalopride op de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine gemeten werd in weefsels gesneden in de richting van de circulaire spierlaag (deze bevatten naast circulaire spiermateriaal ook enig longitudinaal spiermateriaal en de myenterische plexus; "volledig" weefsel), als ook van strips enkel bestaande uit geïsoleerde circulaire spieren en hun zenuwuiteinden, stelden we vast dat prucalopride de elektrisch geïnduceerde vrijstelling van [^{3}H]-acetylcholine doet toenemen; de 5-HT$_4$-receptor antagonist GR113808 neutraliseerde dit effect, wat overeenstemt met de aanwezigheid van stimulerende 5-HT$_4$-receptoren op de cholinerge zenuwuiteinden naar de circulaire spierlaag in het menselijke colon. Wanneer de invloed van prucalopride op elektrisch geïnduceerde cholinerge contracties in "volledig" weefsel strips onderzocht werd, waren de contracties ofwel toegenomen, niet beïnvloed of zelfs verminderd. Een wisselend evenwicht tussen de stimulerende 5-HT$_4$-receptoren op de cholinerge zenuwen, beschreven in deze thesis, en de remmende 5-HT$_4$-receptoren op de gladde spiercellen in het menselijk colon, eerder beschreven, is waarschijnlijk de verklaring.

In het algemeen tonen onze resultaten aan dat de maagfundus van het varken een goed model is om de maagfundus van de mens te bestuderen. Zowel NO als VIP werken acetylcholine enkel postsynaptisch tegen op dit niveau van het GI kanaal. De cholinerge zenuwcellen van de maagfundus van het varken en de mens bevatten presynaptische remmende muscarine-autoreceptoren en α_2-adrenoceptoren. Presynaptische stimulerende 5-HT$_4$-receptoren zijn aanwezig op de cholinerge zenuwuiteinden naar de circulaire spierlaag van de maagfundus alsook van het colon van de mens.